PCT

Date: July 28, 2005 I hereby certify that, on the date indicated above, I deposited this paper with identified attachments and/or fee with the U.S. Postal Service and that it was addressed for delivery to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 by "First Class Mail" service.

Donald S. Prater — Donald S. Prater
Name (Print) — Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Chano et al.	Examiner:	Unassigned
Application No.:	10/516,558	Group Art Unit:	Unassigned
Filed:	November 30, 2004	Confirmation No.:	Unassigned
Docket No.:	3190-070	Customer No.:	33432

For: RB1 GENE INDUCED PROTEIN (RB1CC1) AND GENE

RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

July 28, 2005

Dear Sir:

In response to the Notification of Missing Requirements Under 35 U.S.C. 371 in the United States Designated/Elected Office dated July 11, 2005, it is respectfully submitted that the English translation of the application, the declaration of the inventors, and a copy of the "sequence listing" in computer readable form was previously submitted on January 25, 2005, via Express mail service, as evidenced by the enclosed copy of applicant's PTO date-stamped post card. A copy of the previous response (except a copy of the diskette) is also enclosed. January 25, 2005 is the date that should be reflected in the file for satisfying all requirements under 35 U.S.C. § 371.

In the event that any fees are due with this paper, please charge Deposit Account No. 50-0925.

Respectfully submitted,

Luke A. Kilyk
Reg. No. 33,251

Docket No.: 3190-070
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UNITED STATES PATENT AND TRADEMARK OFFICE

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U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
10/516,558	Tokuhiro Chano	3190-070

INTERNATIONAL APPLICATION NO.	
PCT/JP03/00882	
I.A. FILING DATE	PRIORITY DATE
01/30/2003	

Kilyk & Bowersox
53 A East Lee Street
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CONFIRMATION NO. 2830

371 FORMALITIES LETTER

OC0000000016489947

Date Mailed: 07/11/2005

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as a Designated / Elected Office (37 CFR 1.495).

- Copy of the International Application filed on 11/30/2004
- Copy of the International Search Report filed on 11/30/2004
- Copy of IPE Report filed on 11/30/2004
- Biochemical Sequence Listing filed on 11/30/2004
- U.S. Basic National Fees filed on 11/30/2004
- Priority Documents filed on 11/30/2004

Docketed
Due Date 9/11/05 & 9/30/05
Dkt No 3190-070
By JMB

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Translation of the application into English. Note a processing fee will be required if submitted later than 30 months from the priority date. 7/30/05
- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.
- A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000). Applicant must provide an initial computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). If applicant desires the sequence listing in the instant application to be identical with that of another application on file in the U.S. Patent and Trademark Office, such request in accordance with 37 CFR 1.821(e) may be submitted in lieu of a new CRF.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 32 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

For questions regarding compliance to 37 CFR 1.821-1.825 requirements, please contact:

- **For Rules Interpretation, call (571) 272-0951**
- **For Patentin Software Program Help, call Patent EBC at 1-866-217-9197 or directly at 703-305-3028 / 703-308-6845 between the hours of 6 a.m. and 12 midnight, Monday through Friday, EST.**
- **Send e-mail correspondence for Patentin Software Program Help @ ebc@uspto.gov**

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

*A copy of this notice **MUST** be returned with the response.*

PATRICIA A BOOKER

Telephone: (703) 308-9140 EXT 204

PART 1 - ATTORNEY/APPLICANT COPY

U.S. APPLICATION NUMBER NO.	INTERNATIONAL APPLICATION NO.	ATTY. DOCKET NO.
10/516,558	PCT/JP03/00882	3190-070

FORM PCT/DO/EO/905 (371 Formalities Notice)

U.S. Patent Application No. 10/516,558 Docket No. 3190-070
Filed: November 30, 2004 Applicant: CHANO et al.
Entitled: RB1 GENE INDUCED PROTEIN (RB1CC1) AND GENE

Papers filed herewith on: January 25, 2005
Transmittal Letter Concerning Filing Under 35 U.S.C. 371; English translation of the International Application; Preliminary Amendment (9 pages), Statement under 3 C.F.R. § 1.821, Computer-readable diskette; Executed Declaration; Information Disclosure Statement; Form PTO-1449, 4 documents, and Credit Card Payment Form.

Express Mail Label: EV567259572US
COMMISSIONER FOR PATENTS
Receipt is hereby acknowledged of the papers filed as indicated in connection with the above-identified case

LAK/khb

PTO-1390 (Rev. 12-2004)
Approved for use through 03/31/2007. OMB 0651-0021
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
3190-070	10/516,558

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP03/00882	January 30, 2003	June 3, 2002

TITLE OF INVENTION: RB1 GENE INDUCED PROTEIN (RB1CC1) AND GENE

APPLICANT(S) FOR DO/EO/US: Tokuhiro CHANO, Hidetoshi OKABE, and Shiro IKEGAWA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [ ] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [x] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371 (f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected (Article 31).
5. [ ] A copy of the International Application as filed (35 U.S.C. 371 (c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [x] An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. [x] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [x] Amendments to the claims of the International Application under PCT Article 34 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [x] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 34 (35 U.S.C. 371(c)(3))
9. [x] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [x] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [x] A preliminary amendment.
14. [ ] An Application Data Sheet under 37 CFR 1.76
15. [ ] A substitute specification.
16. [ ] A power of attorney and/or address change letter.
17. [x] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 – 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [ ] Other items or information:

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/516,558	PCT/JP03/00882	3190-070

21. [x] The following fees are submitted:		
[ ] a) Basic national fee $300.00	$ 0.00	
[ ] b) Examination fee $200.00	$ 0.00	
[ ] c) Search fee $500.00	$ 0.00	
TOTAL OF ABOVE CALCULATIONS = $ 0.00	$ 0.00	
[ ] Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing or computer program listing filed in an electronic medium). The fee is **$250.00** for each additional 50 sheets of paper or fraction thereof.		
Total Sheets: 87 - 100 = / Extra sheets: /50 = / Number of each additional 50 or fraction thereof (round **up** to a whole number): 0 x $250.00	$ 0.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than Months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	26 - 20 =	6	x	**$50.00**	$ 300.00
Independent claims	1 - 3 =	0	x	**$200.00**	$ 0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+	**$360.00**	$

TOTAL OF ABOVE CALCULATIONS =	$ 300.00
[ ] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.	$ 0.00
SUBTOTAL =	$ 300.00
Processing fee of **$130.00** for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)).	$ 0.00
TOTAL NATIONAL FEE =	$ 300.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$ 0.00
TOTAL FEES ENCLOSED =	~~$ 300.00~~
Amount to be: Refunded	$
Amount to be Charged	$

COPY

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [redacted] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0925.

d. [redacted] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

KILYK & BOWERSOX, P.L.L.C.
53 A East Lee Street
Warrenton, VA 20186

Phone (540) 428-1701 - Facsimile (540) 428-1720

Luke A. Kilyk
SIGNATURE:

Luke A. Kilyk
NAME

33,251
REGISTRATION NUMBER

Date: January 25, 2005 Label No. EV567259572US I hereby certify that, on the date indicated above, I deposited this paper with identified attachments and/or fee with the U.S. Postal Service and that it was addressed for delivery to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 by "Express Mail Post Office to Addressee" service.

Donald S. Prater / Kim Blum
Name (Print) Signature

RB1 GENE-INDUCED PROTEIN (RB1CC1) AND GENE

Technical Field

The present invention relates to a novel protein and polypeptide (hereunder, referred to as "novel protein RB1CC1") that can induce expression of a tumor-suppressor gene (retinoblastoma gene: RB1 gene). More specifically, the present invention relates to a polypeptide having all or a part of an amino acid sequence of a novel protein, a nucleic acid (hereunder, referred to as "RB1CC1 gene") coding for the polypeptide, a recombinant vector containing the nucleic acid, a transformant that was transformed with the recombinant vector, a method for producing a peptide or polypeptide using the transformant, an antibody against the peptide or polypeptide, a method of screening for compounds that utilizes these, the screened compounds, an activity-inhibiting compound or activity-enhancing compound that acts on the polypeptide or the nucleic acid, a pharmaceutical composition relating to these, and a method of testing or diagnosing a disease relating to these as well as a reagent.

Background of the Invention

Multidrug resistance (MDR) that is resistance to treatment with anticancer agents is a major barrier to the successful treatments of cancer. While current

understanding of factors that contribute to origins of MDR is limited, it is considered that P-glycoprotein that is a product of an MDR-associated gene (MDR1 gene) is involved in several cancers. It is also known that in other cancers expression of P-glycoprotein correlates inversely with emergence and metastasis of the cancer. It is considered that these different effects of P-glycoprotein are subject to suppression by different gene products or conduct different interactions. The identification of genes associated with MDR is essential in order to clarify these phenomena.

Summary of the Invention

A problem to be solved by the present invention is to discover a gene associated with multidrug resistance to anticancer agents as described above and the gene product thereof. More specifically, an object of the present invention is to provide a novel protein and polypeptide (novel protein RB1CC1) that can induce expression of the tumor-suppressor gene (retinoblastoma gene: RB1 gene). Another object of the present invention is to provide the nucleic acid (hereunder, "RB1CC1 gene") coding for all or the part of the amino acid sequence of the novel protein, and the method for producing the protein or polypeptide (novel protein RB1CC1) using genetic engineering techniques. A further object of the present invention is to provide the

antibody against the polypeptide derived from the novel protein RB1CC1. Other objects of the present invention are to conduct screening for an inhibitor, antagonist, or activator for actions of the novel protein RB1CC1 utilizing the aforementioned substances, to provide screened compounds, and to provide the pharmaceutical composition for use in treatment of multidrug resistance (MDR) that is resistance to treatment with anticancer agents utilizing these. Another problem to be solved by the present invention is to provide the method for diagnosing a cancer cells or cancer by testing for the novel protein and polypeptide (RB1CC1 protein) that can induce expression of the tumor-suppressor gene (retinoblastoma gene: RB1 gene) or the nucleic acid (hereunder, "RB1CC1 gene") coding for all or a part of the amino acid sequence of the protein, that were clarified in the present invention. A further object of the present invention is to provide nucleic acid primers that can amplify a nucleic acid coding for all or the part of the amino acid sequence of the protein, and to provide the method for diagnosing cancer cells or cancer by testing for an amplification product of the nucleic acid using primers. A still further object of the present invention is to provide the antibody that can react with the protein or polypeptide (RB1CC1 protein), as well as an immunological assay method that uses the antibody. A further object of the present invention is to provide an assay reagent or kit

that uses the primers or the antibody to be used in the assay method.

In order to solve the above problems, the present inventors identified a gene expressing differentially in U-2 OS osteosarcoma cells and MDR-variant induced cells and determined the nucleotide sequence thereof and the amino acid sequence encoded by cDNA of the novel protein. Further, in order to verify that a similar protein is present in animals, inventors determined the amino acid sequence of a novel protein in mouse and the amino acid sequence encoded by cDNA of the novel protein. In addition, inventors prepared antibodies that recognize these proteins and conducted immunological assay in addition to assay of expression, mutation, deletion and the like for the gene, and found that expression of the gene and expression of the protein are suppressed in certain kinds of cancer cells, thereby completing the present invention.

That is, the present invention comprises the following:

1. A protein or polypeptide which is present in the nucleus of human or animal cell and which has a function that can induce a transcription factor function and/or expression of retinoblastoma gene (RB1 gene) or a gene product thereof.

2. The human protein according to the above 1, which is a polypeptide or protein selected from the group consisting

of: (1) a polypeptide or protein represented by an amino acid sequence described in SEQ ID No: 1 in the sequence listing; (2) a polypeptide containing an amino acid sequence comprising at least five amino acids of the amino acid sequence of the said polypeptide or protein; (3) a polypeptide or protein having homology of at least approximately 70% at the amino acid sequence level with the said polypeptide or protein; and (4) a protein or polypeptide having a mutation or induced mutation such as a deletion, substitution or addition of one to several amino acids relative to the amino acid sequence of the polypeptide or protein according to any one of the preceding (1) to (3).

3. The animal protein according to the above 1 that is a protein derived from mouse, which is a polypeptide or protein selected from the group consisting of: (1) a polypeptide or protein represented by an amino acid sequence described in SEQ ID No: 2 in the sequence listing; (2) a polypeptide comprising at least five amino acids of the amino acid sequence of the said polypeptide or protein; (3) a polypeptide or protein having homology of at least approximately 70% at the amino acid sequence level with the said polypeptide or protein; and (4) a protein or polypeptide having a mutation or induced mutation such as a deletion, substitution or addition of one to several amino acids relative to the amino acid sequence of the said polypeptide or protein according to any one of the preceding (1) to (3).

4. A nucleic acid coding for the polypeptide or protein according to any one of the above 1 to 3, or a complementary strand thereof.

5. A nucleic acid hybridizing under stringent conditions with the nucleic acid or the complementary strand thereof according to the above 3.

6. A nucleic acid represented by a base sequence comprising at least 15 consecutive bases of the base sequence of a nucleic acid described in SEQ ID No: 3 to 4 in the sequence listing or a complementary strand thereof, wherein a polypeptide expressed by transcription of the nucleic acid is the polypeptide according to any one of the above 1 to 3.

7. A recombinant vector containing the nucleic acid according to any one of the above 4 to 6.

8. A transformant that was transformed with the recombinant vector according to the above 7.

9. A method for producing the polypeptide or protein according to any of the above 1 to 3, comprising a step of culturing the transformant according to the above 8.

10. Nucleic acid primers represented by SEQ ID Nos: 5 to 132 in the sequence listing, which hybridize under stringent conditions with the nucleic acid or the complementary strand thereof according to any one of the above 4 to 6.

11. An antibody that immunologically recognizes the polypeptide or protein according to any one of the above 1 to 3.

12. A method of screening for compounds that inhibit or enhance a function that can induce transcription factor activity of the polypeptide or protein and/or expression of RB1 gene according to any of the above 1 to 3, wherein the method uses at least one member of the group consisting of the polypeptide or protein according to any one of the above 1 to 3 and the antibody according to the above 11.

13. A method of screening for compounds that interact with the nucleic acid according to the above 4 or 6 to inhibit or enhance expression of the nucleic acid, wherein the method uses at least one member of the group consisting of the nucleic acid according to any one the above 4 to 6, the vector according to the above 7, the transformant according to the above 8, and the nucleic acid primers according to the above 10.

14. A compound that was screened by the screening method according to the above 12 or 13.

15. A compound that inhibits or enhances a function that can induce transcription factor activity and/or expression of RB1 gene of the polypeptide or protein according to any of the above 1 to 3.

16. A compound that interacts with the nucleic acid according to any one of the above 4 to 6 to inhibit or enhance expression of the nucleic acid.

17. A pharmaceutical composition for use in treatment of multidrug resistance that is resistance to treatment with anticancer agents, wherein the pharmaceutical composition

comprises at least one member of the group consisting of the polypeptide or protein according to any of the above 1 to 3, the nucleic acid according to any one of the above 4 to 6, the vector according to the above 7, the transformant according to the above 8, the nucleic acid primers according to the above 10, the antibody according to the above 11, and the compound according to any one of the above 14 to 16.

18. A method of testing or diagnosing a disease related with expression or activity of the polypeptide or protein according to any of the above 1 to 3, wherein the method comprises a step of conducting analysis employing (a) a nucleic acid encoding the polypeptide or protein and/or (b) the polypeptide or protein in a sample, as a marker.

19. The method of testing or diagnosing according to the above 18, which is a method of testing cancer cells or a method of diagnosing a cancer.

20. The method according to the above 18 or 19 which examines expression, increase, decrease, deletion or the like of all or a part of the polypeptide or protein according to any of the above 1 to 3, wherein the method uses the antibody according to the above 11.

21. The method according to the above 18 or 19 which examines expression, mutation, deletion or insertion or the like of all or a part of a gene encoding the polypeptide or protein according to any of the above 1 to 3 through a step of

amplifying a gene encoding the polypeptide or protein according to any of the above 1 to 3 using at least one of nucleic acid primers according to the above 10.

22. The method according to any of the above 18 to 21, wherein the method combines examination of expression, increase, decrease, mutation, deletion or insertion or the like of all or a part of the tumor-suppressor gene retinoblastoma gene (RB1 gene) or the gene product thereof (RB1 protein).

23. The method according to any of the above 18 to 22, wherein the method combines examination of expression, increase, decrease, mutation, deletion or insertion or the like of all or a part of multidrug resistance gene (MDR1 gene) or the gene product thereof (MDR1 protein: P-glycoprotein).

24. The method according to any of the above 18 to 23, wherein the method combines examination of expression, increase, or decrease or the like of all or a part of the cell proliferation marker, Ki-67 protein.

25. A method that examines drug sensitivity of a cancer cell using the method according to the above 23.

26. A reagent and a kit for assay or diagnosis, for use in the method according to any of the above 18 to 25.

Brief Description of Drawings

Figure 1 shows photographs of Northern blots that examined the relation between expression of human RB1CC1 gene and MDR1 gene.

Figure 2 shows a photograph of Western blots and of cellular immunostaining showing that human RB1CC1 protein is present in nucleus.

Figure 3 shows photographs of Western blots and of cellular immunostaining showing that mouse Rb1cc1 protein is present in nucleus.

Figure 4 is a diagram that examined the effect on cell proliferation resulting from treatment with the anticancer agent doxorubicin.

Figure 5 shows photographs of Northern blots that examined the relation between cell proliferation caused by treatment with the anticancer agent doxorubicin and RB1CC1 gene expression and RB1 gene expression.

Figure 6 is a photograph of electrophoresis of RT-PCR products that examined the relation between RB1CC1 gene expression and RB1 gene expression in various cancer cells.

Figure 7 shows photographs of Northern blots that examined the relation between RB1CC1 gene expression and RB1 gene expression in various human organs.

Figure 8 is a photograph of a Northern blot that examined the relation between RB1CC1 gene expression and RB1 gene expression in various mouse organs.

Figure 9 is a photograph of electrophoresis of RT-PCR products that examined the effect on RB1 gene expression caused by introduction of RB1CC1 gene.

Figure 10 is a diagram showing results obtained after

testing the effect on transcriptional activity of RB1 gene promoter region caused by RB1CC1 gene induction.

Figure 11 is a photograph of results obtained after testing loss of heterozygosity of RB1CC1 gene locus in a variety of primary breast cancers.

Figure 12 shows a photograph of electrophoresis of RT-PCR products that examined mutation of RB1CC1 gene in primary breast cancers, and a view showing the results of gene sequence analysis.

Figure 13 shows photographs of Western blots that examined expression of RB1CC1 protein and RB1 protein in primary breast cancers.

Figure 14 shows photographs of immunohistological staining that examined expression of RB1CC1 protein and RB1 protein in primary breast cancers.

Figure 15 shows diagrams illustrating the correlation between RB1CC1 as a stain indicator and Ki-67 and RB1.

Detailed Description of the Invention

(Novel protein RB1CC1)

The cDNA of the nucleic acid encoding the novel protein RB1CC1 provided according to the present invention was obtained by identifying a gene expressing differentially in U-2 OS osteosarcoma cells and MDR-variant induced cells, conducting amplification employing U-2 OS mRNA as a template using nucleic acid primers described in SEQ ID Nos: 5 to

37 in the sequence listing, and determining the amino acid sequence coded for by cDNA of the novel protein and the base sequence, to thereby obtain the cDNA as a substance having a novel amino acid sequence. The cDNA of novel protein RB1CC1 of the present invention had a length of 6.6 kb, included an open reading frame (ORF) of 4782 nucleotides, and encoded a protein comprising 1594 amino acids with a molecular weight of 180 kDa.

The novel human protein RB1CC1 had a consensus nuclear localization signal sequence site (lysine-proline-arginine-lysine sequence: KPRK), a leucine zipper motif sequence site, and a coiled-coil structure. It was suggested that the novel human protein RB1CC1 has DNA-binding and transcription functions.

(Novel mouse protein Rb1cc1)

Amplification was conducted employing mRNA of mouse muscle as a template using the nucleic acid primers described in SEQ ID Nos: 53 to 83 in the sequence listing, and the amplification product was analyzed. The obtained cDNA coding for novel mouse protein Rb1cc1 had a chain length of 6518 bp with an open reading frame (ORF) of 4764 bp encoding 1588 amino acids. The novel mouse protein Rb1cc1 gene shared 89% homology with the novel human protein RB1CC1 gene. Similarly to the human protein, novel mouse protein Rb1cc1 had a consensus nuclear localization signal sequence site

(lysine-proline-arginine-lysine sequence: KPRK), a leucine zipper motif sequence site, and a coiled-coil structure. It was suggested that mouse novel protein Rb1cc1 also has DNA-binding and transcription functions.

(Function of novel protein and gene)

To investigate the role of RB1CC1 gene of the present invention in MDR, RB1CC1 gene expression was compared for cases in which doxorubicin treatment was conducted for parental U-2 OS cells, MDR variants of U-2 OS cells (U-2 OS/DX580), and U-2 OS cells introduced with MDR1 gene (U-2/DOXO35), whereby it was found that in the parental U2 OS cells and control cells introduced with a gene (U-2/Neo8) doxorubicin lowered expression of the RB1CC1 gene and induced cell death. In contrast, in the MDR variants of U-2 OS cells, doxorubicin treatment did not exhibit an inhibitory effect on the expression level of RB1CC1 gene, cell lifetime, or cell proliferation, and in cells with the MDR1 gene the RB1CC1 gene expression was increased. In these cells, RB1CC1 gene expression and RB1 gene expression correlated, and expression of both genes sustained the proliferation of these cells.

To examine the relation between expression of RB1 gene and the RB1CC1 gene of the present invention, expression of both genes in 5 kinds of MDR-variants of U-2 OS human osteosarcoma cells and 24 kinds of human tumor cells (10

kinds of osteosarcoma, 4 kinds of lung cancer, 7 kinds of breast cancer, 3 kinds of blood cancer) was examined, whereby it was found that RB1CC1 gene expression strongly correlated with RB1 gene expression in all of the cells. Expression of RB1CC1 gene and RB1 gene also showed a similar correlation in Northern blot analysis of nonneoplastic tissue.

Further, exogenous expression of the RB1CC1 gene of the present invention increased RB1 gene expression in K562 cells and Jurkat cells. Expression of MDR1 gene could not be detected in these cells. Induction of RB1CC1 gene also stimulated transcriptional activity of RB1 gene promoter. Introduction of the RB1CC1 gene raised expression of RB1 gene through the stimulated activity of the RB1 gene promoter.

Considering the amino acid sequence of the novel protein RB1CC1, the nuclear locality thereof, and the expression pattern thereof, there is a possibility that the RB1CC1 gene of the present invention is a transcription factor that enhances RB1 gene expression directly or indirectly through a molecular intermediate. While analysis of promoter sequences of RB1 genes derived from human and mouse indicates the possibility of the presence of a constitutive transcription factor such as Sp1 or ATF, a transcription factor that directly regulates RB1 gene expression is not known. In about 80% of human cancers, molecules that are present in the RB1 gene pathway are

associated with the mechanism of carcinogenesis, and dysregulation of the RB1 gene plays an important role in the cancer of many people.

As shown in Table 1, human and mouse RB1CC1 genes of the present invention both contain 24 exons and 23 introns, and length 74 kb or more and 57 kb or more in human and mouse, respectively. A translation initiation position is present at the site of exon 3. The structure of the gene in mouse was clarified using primers set forth in SEQ ID Nos: 84 to 132 of the sequence listing. When we investigated the localization sites of the gene on a chromosome, we found that the gene is present at 8q11.2 on the chromosome 8 in human and at 1A2-4 on the chromosome 1 in mouse.

Table1. Structure of RB1CC1 gene

Exon No.	Exon nucleic acid strand length (bp) human	Exon nucleic acid strand length (bp) mouse	Intron No.	Intron nucleic acid strand length (kb) human	Intron nucleic acid strand length (kb) mouse	Human Sequence: receptor sequence in splicing		Human Sequence: donor sequence in splicing	
1	358	296	1	9.1	11.2			GCGTTGCCGG	gtaagtgtcg
2	115	110	2	1.3	1.8	tcttttccag	TTTTCTGAGT	GTGCCTGACG	gtaagtcaca
3	122	115	3	1.4	3.5	tttcttctag	TAACTGTATC	CAGTGCAAAC	gtaagttgta
4	127	127	4	0.2	0.1	tttttgaag	TGTGGCAGAC	TGCTGGGACG	gtaggtattc
5	171	171	5	7.0	3.8	aaaaatatag	GATACAAATC	GCTTGCATTG	gtaagatata
6	203	203	6	2.1	1.3	ttcaatatag	GAAATGTATG	AACTTACTCA	gtatgtttgc
7	430	427	7	5.7	3.8	gtattttaag	TTTAGGAACT	TATGAGCAGG	gtaagtaacg
8	171	171	8	6.3	0.5	tgtcatttag	CTTGATCCAA	GCTTGCTCAG	gtacctattt
9	185	185	9	0.3	0.2	tttctcaaag	GGATTTTTAG	TCAGACTGAA	gtaagtgatt
10	187	187	10	0.1	0.1	tattctctag	GTGGTGTTGC	CTACAGGGAG	gtatgcaagt
11	82	82	11	0.3	0.1	cctcttctag	TGGGCTGGTG	AAATTATTTA	gtaagtgttc
12	62	62	12	1.6	1.6	ctttatacag	GGAAGTCTTT	TTCCTTTTGT	gtatgtattt
13	104	104	13	0.8	0.3	tttgstacag	ACTCAAAAGC	CATTCCTCAG	gtaaatgtca
14	127	127	14	0.1	0.1	tctgtttcag	GGTTCCCTTA	TGAACAAAAG	gcaaattcaa
15	1801	1892	15	10.1	10.0	tgttttccag	GCATCTGTGA	TAGCAAAAAG	gtaagaatta
16	166	166	16	2.9	1.6	aatttgtaag	TCCTGCCATT	GGAACAACAG	gtctgtatct
17	109	109	17	0.1	0.1	cttgttccag	ACCAATTTTA	CGGGATAAAG	gtttgtactg
18	241	241	18	6.3	1.1	tgtccttcag	ATTTGATAGA	TGTCTGTACA	gtaagtatgg
19	55	49	19	1.0	1.0	tcacttttag	AGAAAATATT	GTTAGAACGA	gtaagtaaat
20	48	48	20	4.4	3.0	ccacctgcag	ACATTGCAAT	TCAAAGACTG	gtaagatttt
21	59	59	21	2.3	2.1	tttttttag	ATGTCTCAGA	CTATTAGAGA	gtaagtattt
22	137	137	22	3.5	2.0	ctttattcag	TTTTCAGGTG	GGTGAGGGTG	gtaagtgtca
23	71	71	23	0.8	1.6	atttcattag	CTTCAGGTGC	AGCCAAAAAG	gtaaaaacga
24	1401	1379				tccctcttag	GCACAAAACA		

Exon sequernces are shown in upper case letters, and intron sequences are shown in lower case.

In order to detect mutations of RB1CC1 gene of the present invention, the RB1CC1 gene was analyzed using cDNA prepared from 35 cases of primary breast cancer, whereby 9 kinds of mutation were verified in 7 of cancers. There were lacks at exons 3 to 24 in all of 9 kinds of mutation, and the fragmented novel protein RB1CC1 had lost its consensus nuclear localization signal sequence site, leucine zipper motif sequence site and coiled-coil structure, and did not have functions of the fundamental novel protein RB1CC1.

Two of primary breast cancers (MMK 3 and 6) showed compound heterozygous lacks in both alleles, and it is predicted that a clearly fragmented novel protein RB1CC1 can be obtained from RB1CC1 gene with a lack. In MMK 6, there were lacks at exons 3 to 24 (nucleotides 534-5322) and exons 9 to 23 (nucleotides 1757-5187), with the respective frameshifting at codons 4 and 411. In MMK 3, there were lacks at exons 3 to 24 (nucleotides 535-5324) and exons 5 to 11 (nucleotides 849-2109), with termination occurring at codon 4 in the former, and a frame shift caused at codon 109 in the latter to result in obtainment of a protein fragment comprising 122 amino acids. Although irregular products corresponding to respective lack mutations were detected in PCR of genome DNA of cancer samples, mutations were not observed in DNA of embryonic cells, revealing that these mutations occur in somatic cells. The novel protein

RB1CC1 was not detected in these cancers, and RB1 protein was absent in MMK 6 and was significantly less abundant than normal in MMK 3. There was no loss of heterozygosity at the RB1 loci on the chromosome in either case. In the cancer samples (MMK 12 and 29) without mutation of the RB1CC1 gene, both the novel protein RB1CC1 and RB1 protein were present. This suggests that inactivated mutation of the RB1CC1 gene causes RB1 gene expression to be insufficient and promotes dysregulation of the RB1 gene pathway, to cause canceration.

In other five breast cancers, (MMK 1, 15, 31, 38 and 40) also, lacks were detected in RB1CC1 gene that generated a protein fragment without function. These mutations were all heterozygotes, with loss of heterozygosity also present at the RB1CC1 loci, and since there was no expression of RB1CC1 gene in each of the cases, it was suggested that loss of function had occurred in both alleles. Expression of RB1 protein in these cancers was clearly reduced in comparison to cases (MMK 12 and 29) without mutation of RB1CC1 gene and RB1 gene. Loss of heterozygosity at the RB1 loci was not observed in these 5 cancers (MMK 1, 15, 31, 38, and 40).

Homozygous inactivation of the RB1CC1 gene of the present invention is associated with genesis of breast cancer. Lack mutations of the RB1CC1 gene that generated fragments of the novel protein RB1CC1 that clearly had no function were observed in approximately 20% of primary breast cancers

examined. Two of these cancers showed plural heterozygous lacks within the RB1CC1 gene, and the remainder showed loss of heterozygosity of the RB1CC1 gene. Although the novel protein RB1CC1 could not be detected in any of seven cancers, protein was expressed in cancers without mutation of the RB1CC1 gene. Irrespective of the fact that there was no loss of heterozygosity at the RB1 loci, in all seven cancers the RB1 protein was either absent or significantly decreased.

The novel protein RB1CC1 performs regulation to increase expression of the RB1 gene, and the RB1CC1 gene functions as a tumor suppressor in breast cancer. Further, abnormality or inactivation of the RB1CC1 gene leads to a decline in expression of RB1 gene, causing genesis and progression of cancer.

As described in the above-mentioned, since expression of the RB1CC1 gene and protein correlate with expression of RB1 gene, a more useful method of diagnosing cancer cells or cancer can be provided by performing tests that combine testing for the RB1CC1 gene and protein of the present invention with testing for expression of the RB1 gene or expression of the protein.

Further, by also combining tests for multidrug resistance gene (MDR1) or the protein thereof, the effect of a pharmaceutical against a cancer or cancer cells can be examined, enabling the provision of an examination method or a diagnostic method that is useful for selecting an

anticancer agent and predicting the effects thereof.

(Polypeptide or protein)

The novel protein of the present invention is a polypeptide or protein comprising an amino acid sequence represented by SEQ ID No: 1 or 2 in the sequence listing. The polypeptide or protein of the present invention may also be selected from polypeptides having a partial sequence of the polypeptide represented by SEQ ID No: 1 or 2 in the sequence listing. The selected polypeptide preferably has homology of about 70% or more, more preferably about 80% or more, and further preferably has homology exceeding about 90% with the polypeptide represented by SEQ ID No: 1 or 2 in the sequence listing. Selection of polypeptides having the homology can be conducted, for example, by taking expression of RB1 gene or RB1 protein as an indicator.

Techniques for determining homology of an amino acid sequence are publicly known in the art and, for example, a method that directly determines the amino acid sequence or a method that first determines a putative base sequence of a nucleic acid and then predicts the amino acid sequence encoded by the base sequence may be used.

For the polypeptide of the present invention, an amino acid sequence selected from polypeptides having a partial sequence of a polypeptide or protein comprising an amino acid sequence set forth in SEQ ID No: 1 or 2 in the sequence

listing can be utilized as a reagent, reference material or immunogen. The subject of the present invention is a polypeptide comprising, as a minimum unit thereof, the amino acid sequence composed of at least 5 amino acids, preferably at least 8 to 10 amino acids or more, and more preferably at least 11 to 15 or more amino acids which can be screened immunologically.

Further, by employing expression of RB1 gene or RB1 protein as the indicator, there can also be provided a polypeptide comprising an amino acid sequence having a mutation or induced mutation such as a deletion, substitution, addition or the like of one to several amino acids relative to the amino acid sequence of a polypeptide specified as described above. Methods for carrying out a deletion, substitution, addition or insertion are publicly known, and, for example, the technique of Ulmer (Science, 219: 666, 1983) can be utilized. These available peptides can also be modified to a degree that is not accompanied by a noticeable change in function, such as modification of constitutive amino groups or carboxyl groups or the like.

Polypeptides of the present invention can be used as they are in a pharmaceutical composition for regulating a function of the novel protein RB1CC1. Further, the polypeptide or protein of the present invention can be used in screening to obtain a compound that can regulate a function of the novel protein RB1CC1, for example, an inhibitor,

antagonist, activator or the like, or an antibody against the novel protein RB1CC1. In addition, a polypeptide or protein of the present invention can also be used as a reagent or reference standard.

(Nucleic acid)

The term "nucleic acid and a complementary strand thereof" of the present invention refers to a nucleic acid set forth in SEQ ID No: 3 or 4 in the sequence listing that codes for an amino acid sequence set forth in SEQ ID No: 1 or 2 in the sequence listing and the complementary strand for the nucleic acid, a nucleic acid hybridizing under stringent conditions with these nucleic acids, and a nucleic acid having a sequence of at least 15 consecutive base sequence derived from these nucleic acids in which a peptide encoded thereby is capable of binding with an antibody against the novel protein RB1CC1. When DNA is taken as a typical example of the nucleic acid, the term "DNA hybridizing under stringent conditions to DNA" refers to DNA that can be obtained by a publicly known method, for example, a method described in Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). Here the term "hybridizing under stringent conditions" refers to, for example, conditions under which a positive hybridization signal is still observed even after heating at 42 °C in a solution of 6 × SSC, 0.5% SDS and 50%

formamide, and washing at 68 °C in a solution of 0.1 × SSC and 0.5% SDS.

The term "nucleic acid of the present invention" refers to a homologous strand and complementary strand selected from information of the nucleic acid set forth in SEQ ID No: 3 or 4 in the sequence listing that encodes an amino acid sequence described in SEQ ID No: 1 or 2 in the sequence listing, and also refers to a nucleic acid sequence comprising a sequence of at least about 15 to 20 nucleotides that correspond to a region of the specified nucleotide sequence, as well as the complementary strand thereof. Determination of this useful nucleic acid sequence can be conducted by simply confirming the expressed protein utilizing a publicly known protein expression system, for example, a cell-free protein expression system, and then screening by employing binding thereof with the antibody against bioactive novel protein RB1CC1 as the indicator. As the cell-free protein expression system, for example, a ribosome system derived from germ or rabbit reticulocyte or the like can be utilized (Nature, 179, 160-161, 1957).

Each of these nucleic acids provide genetic information that is useful for producing the novel protein RB1CC1 of the present invention and the polypeptide or protein of the present invention, and they can be used as primers or probes for detecting mRNA or a nucleic acid such as a gene encoding these, or as antisense oligomers to

regulate gene expression. Further, a nucleic acid of the present invention can also be utilized as a reagent or reference standard relating to the nucleic acid.

(Transformant)

In addition to the cell-free protein expression system described above, by employing genetic recombination techniques using a publicly known host such as *Escherichia coli*, yeast, *Bacillus subtilis*, an insect cell or animal cell, it is possible to provide the novel protein RB1CC1 comprising the present invention and the polypeptide comprising a product derived therefrom.

Transformation can be conducted by applying publicly known means, for example, by transforming the host utilizing a plasmid, chromosome, virus or the like as a replicon. As a more preferable system, a method that conducts integration into the chromosome may be mentioned when considering genetic stability. However, as a simple and convenient method, an autonomous replication system using an extranuclear gene can be utilized. A vector can be selected according to the kind of host, and gene sequences that are objects of expression and gene sequences carrying information relating to replication and regulation can be employed as constituent elements. Constituent elements can be selected according to whether the host is a prokaryotic cell or eukaryotic cell, and a promoter, ribosome binding site, terminator, signal

sequence, enhancer and the like can be combined according to a publicly known method and used.

The transformant can be used to produce the polypeptide of the present invention by culturing the transformant after selecting optimal conditions from publicly known culture conditions for the respective hosts. While culturing may be conducted by employing as an indicator the physiological activity of the novel protein RB1CC1 to be expressed and produced and a polypeptide comprising the product derived therefrom, in particular, RB1 gene inducing activity or DNA-binding transcription factor activity, it is generally conducted by subculture or batch culture employing the quantity of transformant in the medium as an indicator.

(Recovery of the novel protein RB1CC1 and product derived therefrom)

Recovery from the culture medium of the novel protein RB1CC1 and the polypeptide comprising the product derived therefrom can be conducted by carrying out purification and recovery that combines techniques such as a molecular sieving, an ion column chromatography, an affinity chromatography employing binding with the antibody against the novel protein RB1CC1 as the indicator, or by a fractionation technique using alcohol or ammonium sulfate or the like that is based on difference in solubility.

(Antibody)

An antibody can be prepared by screening for an antigenic determinant of the novel protein RB1CC1 of the present invention and the polypeptide comprising the product derived therefrom. The antigenic determinant is composed of at least five amino acids, and more preferably at least 8 to 10 amino acids. The amino acid sequence need not necessarily be homologous with SEQ ID No: 1 or 2 in the sequence listing, and it is sufficient that the sequence is a site that is exposed to outside of the tertiary structure of the protein. If the exposed site is a discontinuous site, it is also effective that the amino acid sequence that is continuous with respect to the exposed site. The antibody is not particularly limited as long as it immunologically recognizes the novel protein RB1CC1 and the polypeptide comprising the product derived therefrom. The presence or absence of the recognition can be determined by a publicly known antigen-antibody binding reaction.

Production of the antibody can be conducted by inducing immunity such as humoral response and/or cellular response in an animal using the novel protein RB1CC1 of the present invention and the polypeptide comprising the product derived therefrom by itself or in a state in which it is bonded with a carrier, in the presence or absence of an adjuvant. The carrier is not particularly limited as long as the carrier itself does not produce a deleterious effect on a host, and

examples thereof include cellulose, polymerized amino acid, and albumin. As an animal to be immunized, mouse, rat, rabbit, goat, horse or the like is preferable. A polyclonal antibody can be obtained by a publicly known method for recovering antibody from serum.

Production of a monoclonal antibody can be carried out by recovering antibody-producing cells from the animal that has undergone the aforementioned immunization and introducing transformation means to publicly known constantly proliferating cells.

The polyclonal or monoclonal antibody can be bonded directly with the novel protein RB1CC1 of the present invention to enable control of the activity thereof, and control of expression of the novel protein RB1CC1 and RB1 gene or protein can be easily performed. Therefore, the antibody is useful for treating or preventing a disease with which the RB1 gene product and the novel protein RB1CC1 are associated.

(Screening)

According to the novel protein RB1CC1 and the polypeptide comprising the product derived therefrom that were prepared as described above, the nucleic acid encoding these and a complementary strand thereof, the cell transformed based on information of these amino acid sequences and base sequences, and the antibody that

immunologically recognizes the novel protein RB1CC1 and the polypeptide comprising the product derived therefrom, by use of a single means or by combining a plurality of means, there can be provided means effective in screening for binding with the novel protein RB1CC1 and the polypeptide comprising the product derived therefrom, a function of the novel protein RB1CC1, or an inhibitor or activator of expression of the novel protein RB1CC1. More specifically, there can be provided a method of screening for compounds that inhibit or enhance expression of the polypeptide or protein and the RB1 gene or protein of the present invention by using at least one member of the group consisting of the polypeptide of the present invention and the antibody of the present invention. There can be provided a method of screening for compounds that interact with the nucleic acid of the present invention to inhibit or enhance expression of the nucleic acid by using at least one member of the group consisting of the nucleic acid of the present invention, vector of the present invention, transformant of the present invention, and antibody of the present invention. There can be provided a method of screening for compounds that inhibit or enhance a function of the polypeptide or protein of the present invention to regulate expression of the RB1 gene or protein by using at least one member of the group consisting of the polypeptide or protein of the present invention and the antibody of the present invention. For example,

screening for the antagonist obtained by drug design based on the tertiary structure of the polypeptide, screening for an expression regulator at the genetic level that utilizes a protein expression system, screening for an antibody recognizing substance utilizing the antibody and the like can be utilized in a publicly known pharmaceutical screening system.

(Compound, pharmaceutical composition)

Compounds obtained by the above-described screening methods can be utilized as candidate compounds for the inhibitor, antagonist, activator or the like that regulates a function of the novel protein RB1CC1 and the polypeptide comprising the product derived therefrom to control expression of RB1 gene or protein. Compounds can also be utilized as candidate compounds for an inhibitor, antagonist, activator or the like for expression of the novel protein RB1CC1 and the polypeptide comprising the product derived therefrom at the genetic level. Examples of aforementioned candidate compounds for an inhibitor, antagonist, activator or the like include a protein, a polypeptide, a polypeptide without antigenicity, and a low molecular weight compound, and a low molecular weight compound is preferred.

Candidate compounds that were screened in the above manner can be selected in consideration of a balance between biological usefulness and toxicity to be prepared as

pharmaceutical compositions to be used for treatment of osteosarcoma, leukemia or a tumor originating from the mammary gland, prostate gland, lung, or colon or the like. Further, the novel protein RB1CC1 comprising the present invention and the polypeptide comprising the product derived therefrom, nucleic acids encoding these and complementary strands thereof, vectors containing these base sequences, and antibodies that immunologically recognize the novel protein RB1CC1 and the polypeptide comprising the product derived therefrom can be used as pharmaceutical means, by themselves, that have an inhibitory, antagonizing or activating function with respect to interaction between the novel protein RB1CC1 and RB1 gene product and are used in treatment of breast cancer, prostrate cancer and the like . Here, the term "breast cancer, prostrate cancer and the like" includes a benign tumor and a malignant tumor, and in this connection, at the time of formulation, publicly known formulation means may be introduced in accordance with the substance for formulation, such as the polypeptide, protein, nucleic acid or antibody.

The novel protein RB1CC1 of the present invention and the polypeptide comprising the product derived therefrom, nucleic acids encoding these and complementary strands thereof, vectors containing these base sequences, and antibodies that immunologically recognize the novel protein RB1CC1 and the polypeptide comprising the product derived

therefrom can be used as means for testing or diagnosing a disease with which expression of the polypeptide of the present invention or the activity thereof is related, such as a disease relating to expression of the novel protein RB1CC1 of the present invention or interaction with RB1 gene or the product thereof. In particular, they are useful as means for examination and diagnosis such as a diagnostic marker and/or reagent or the like for breast cancer, prostrate cancer and the like. Diagnosis can be conducted by utilizing interaction or reactivity with the nucleic acid sequence encoding the novel protein RB1CC1 to determine the abundance of a nucleic acid sequence of interest, and/or determine the biodistribution for the novel protein RB1CC1, and/or determine the abundance of the novel protein RB1CC1 in a test sample. More specifically, testing can be conducted utilizing the novel protein RB1CC1 as the diagnostic marker. As a method of determination, a publicly known antigen-antibody reaction system, enzyme reaction system, PCR reaction system or the like may be used. Further, a reagent kit or the like used in a method of examination and diagnosis is also included.

(Examples)

The present invention is described in further detail hereunder on the basis of examples, however, the present invention is not limited by the following examples.

(Example 1 cDNA of human RB1CC1)

In order to identify genes involved in MDR, we found a gene that expresses differentially in U-2 OS osteosarcoma cells and MDR-variant induced cells, to thereby identify a novel human gene. The gene was cloned using the set of primers (CC1-S1 and CC1-AS1) set forth in SEQ ID Nos: 5 and 26 and the set of primers (CC1-S2 and CC1-AS2) set forth in SEQ ID Nos: 6 and 25 in the sequence listing, and the nucleic acid sequence thereof was then determined using the primers set forth in SEQ ID Nos: 7 to 24. Further, the cDNA sequences at the 5'- and 3'-ends were identified using a commercially available rapid amplification kit for cDNA end sequences (RACE kit, manufactured by Roche) and the primers set forth in SEQ ID Nos: 27 to 37. The DNA and the amino acid sequence encoded thereby were analyzed using DNAsis Version 3.2 Sequence Analyzer (manufactured by Hitachi Software Engineering Co.) and PSORT II (http://www.yk.rim.or.jp/~aisoai/molbio-j.html). Results showed that the cDNA had a length of 6.6 kb including an open reading frame (ORF) of 4782 nucleotides, encoding a protein comprising 1594 amino acids with a molecular weight of 180 kDa.

(Example 2 cDNA of mouse Rb1cc1)

The mRNA of mouse muscle was employed as a template

for amplification by RT-PCR, and cloning was then conducted using the set of primers (MCC1-S1 and MCC1-AS1) set forth in SEQ ID Nos: 53 and 73 and the set of primers (MCC1-S2 and MCC1-AS2) set forth in SEQ ID Nos: 54 and 72 in the sequence listing. The nucleic acid sequence was determined using primers set forth in SEQ ID Nos: 55 to 71 in the sequence listing. The cDNA of novel mouse protein Rb1cc1 was then identified using a similar method to Example 1, with the exception that rapid amplification of the cDNA was conducted using the primers (MCC-ASR1, MCC-ASR2, MCC-ASR3 and INTRON1ASR) set forth in SEQ ID Nos: 74 to 77 in the sequence listing as primers for the 5'-end RACE, and the primers (MCC-SR1, MCC-SR2, MCC3-S3, MCC3-S4, MCC3-AS2 and MCC3-AS3) set forth in SEQ ID Nos: 78 to 83 as primers for the 3'-end RACE. The cDNA encoding novel mouse protein Rb1cc1 has a strand length of 6518 bp including an open reading frame (ORF) of 4764 bp encoding 1588 amino acids. The gene of novel mouse protein Rb1cc1 had homology of 86% at the nucleic acid level and 89% at the protein level with the gene of novel human protein RB1CC1 (see SEQ ID Nos: 1 to 4).

(Example 3 Analysis of MDR1 gene and RB1CC1 gene of the present invention)

Expression levels of RB1CC1 gene and MDR1 gene in parental U2 OS cells and several kinds of MDR-variant cells were analyzed by Northern blotting. A probe hybridizing

between nucleotide numbers 4190 and 4654 of the RB1CC1 gene sequence was used as a probe for analysis of RB1CC1 gene, and a probe hybridizing between nucleotide numbers 834 and 1119 of MDR1 gene was used for MDR1 gene. Probes were used after labeling with α-32P-dCTP in which phosphorus at an alpha position of deoxycytidine-3-phosphate was substituted with a radioactive isotope. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the indicator of mRNA expression. The results showed that the expression levels of both genes correlated inversely (Figure 1).

(Example 4 Preparation of antibody and Western blot analysis)

Three kinds of synthetic polypeptide were prepared which respectively comprised amino acids 642 to 658 (RB1CC-642), 744 to 757 (RB1CC-744) and 1104 to 1118 (RB1CC-1104) of the amino acid sequence of the novel protein RB1CC1 of the present invention. Rabbits were immunized by a conventional method with substances in which a cysteine residue had been introduced at the amino terminus of each polypeptide, and antibody was then obtained. After subjecting nuclear components and cytoplasmic components of U-2 OS cells to SDS-PAGE, respectively, analysis was carried out by Western blotting using the antibody prepared above. Results showed that RB1CC1 protein of a molecular

weight of 180 kDa was present in the nucleus (Figure 2).

After subjecting nuclear components and cytoplasmic components of NIH3T3-3 cells of mouse to electrophoresis in a similar manner, Western blot analysis was conducted using the RB1CC-642 antibody. Detection of stathmin was simultaneously conducted using anti-stathmin rabbit antibody. Results showed that the Rb1cc1 protein is localized in the nucleus, while stathmin is present in cytoplasm. When same cells were subjected to immunocytochemical staining using each antibody and then compared, it was found that while the nucleus was stained with the RB1CC-642 antibody, the cytoplasm was stained with the anti-stathmin rabbit antibody (Figure 3).

Above results showed that the novel protein RB1CC1 of the present invention is present in the nucleus of mammalian cells.

(Example 5 Effect of anticancer agent on expression of RB1CC1 gene of the present invention)

The influence of an anticancer agent was assessed for 4 kinds of cells that were treated with doxorubicin, including parent cells (U-2 OS), MDR variants of U-2 OS cells (U-2 OS/DX580) and U-2 OS cells introduced with the MDR1 gene (U-2/DOXO 35). The effect on cell proliferation in the presence of 450 ng/mL of the anticancer agent doxorubicin was examined. As shown in Figure 4, results indicated that

while cell proliferation was suppressed by the anticancer agent in parental U2 OS cells and control cells introduced with a gene (U-2/Neo8), the anticancer agent had no effect on MDR variants of U-2 OS cells (U-2 OS/DX580) and U-2 OS cells introduced with MDR1 gene (U-2/DOXO 35) and cell proliferation continued for 120 hours or more (Figure 4).

mRNA expression levels of cells that were obtained over time in the above-mentioned experiment were analyzed. Analysis was conducted for the novel gene RB1CC1 gene of the present invention, the RB1 gene and the MDR1 gene, respectively, in the same manner as Example 3 with the exception that expression levels of the RB1 gene were detected using a probe hybridizing to the site at nucleotides 336 to 675 of the nucleotide sequence of human RB1 mRNA. Results are shown in Figure 5. For parental U2 OS cells and control cells introduced with a gene (U-2/Neo8) for which the effect of the anticancer agent was observed, expression of the RB1CC1 gene decreased over time. In contrast, in MDR variants of U-2 OS cells (U-2 OS/DX580) and U-2 OS cells introduced with MDR1 gene (U-2/DOXO 35), expression level of RB1CC1 gene was not inhibited by treatment with doxorubicin, and expression of RB1CC1 gene increased. In these cells, RB1CC1 gene expression and RB1 gene expression correlated (Figure 5).

(Example 6 Expression of RB1 gene and RB1CC1 gene of the

present invention)

The expression of RB1CC1 gene and RB1 gene in various cancer cells was assessed by semi-quantitative RT-PCR. Cell lines used were SARG, IOR/OS9, 10, 14, 15, 18, MOS (these were obtained from surgical samples of advanced human osteosarcoma), Saos-2, HOS, MCF-7, T-47D, BT-20, SK-BR3, ZR75-1, MDA-MB-231, Daudi, Jurkat and K562 (these were purchased from the American Type Culture Collection), NZK-K1 (this was established from breast cancer tissue of a 46-year old female), LK2, QG56, EBC1 and SBC2 (these were provided by Doctor Tatsuhiko Narita of Aichi Cancer Center). 2 μg of RNA was extracted from each cell line, and subjected to 22 to 30 cycles of RT-PCR for amplification. Publicly known primers were synthesized and used as primers for the RB1 gene (Sauerbrey et al., 1996). The combination of primers set forth in SEQ ID Nos: 19 and 20 in the sequence listing (CC1-S and CC1-AS) were used as primers for amplification of RB1CC1. β_2-microglobulin was used as a control. In all of these cells, expression of RB1CC1 gene correlated closely with that of RB1 gene. Figure 6 shows results for one case of normal leukocyte and six cancer cells: T-47D, MCF7, NZK-K1, Daudi, K562 and Jurkat (Figure 6).

(Example 7 Expression of RB1CC1 gene and RB1 gene of the present invention in organs)

Northern blot analysis was conducted for RB1CC1 gene

and RB1 gene expressing in nonneoplastic tissue of human brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung and leukocyte, respectively, using commercially available MTN Blots (manufactured by Clontech). Results are shown in Figure 7. Both genes were expressed strongly in heart and skeletal muscle, while expression was weak in colon, small intestine, lung and leukocyte. However, expression of RB1CC1 gene and RB1 gene correlated. Northern blot analysis was also conducted for Rb1cc1 gene expressing in respective tissues of heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis of mouse. Results are shown in Figure 8. Transcription products of 6.2 kb and 6.8 kb were expressed strongly in heart, while expression was observed to a certain extent in kidney, liver and skeletal muscle. The principal expression in testis was 6.2 kb, while expression was weak in lung and spleen (Figure 7, Figure 8).

(Example 8 Expression of RB1 gene induced by introduction of RB1CC1 gene of the present invention)

Jurkat and K562 cells that had weak expression levels for both RB1CC1 gene and RB1 gene among cells shown in Example 6 were subjected to exogenous introduction of RB1CC1 gene to examine changes in the expression of RB1 gene. A 4.9-kb gene that included the complete coding region of the RB1CC1 molecule was incorporated into pCR3.1-Uni vector

(manufactured by Invitrogen), which was then cloned to prepare an RB1CC1 expression vector (pCR-RB1CC). The thus-prepared expression vector was incorporated into K562 and Jurkat cells to prepare RB1CC1 transformed cells. A control was prepared by incorporating lac Z gene into pCR3.1-Uni vector. Respective expression levels of RB1CC1 gene and RB1 gene in parent cells and transformed cells (cells introduced with RB1CC1 gene) were examined in a similar manner to Example 6. Figure 9 shows the results. Although expression of both RB1CC1 gene and RB1 gene was weak in untransformed cells and cells into which the lac Z gene was incorporated, it was found that in cells incorporated with RB1CC1 gene, the RB1CC1 gene expression was strong as expected and the RB1 gene was also strongly expressed, showing that expression of the RB1 gene was also induced by introduction (exogenous expression) of the RB1CC1 gene (Figure 9).

(Example 9 RB1 gene promoter transcriptional activity of RB1CC1 gene of the present invention)

We examined whether introduction of the RB1CC1 gene enhanced the transcriptional activity of the promoter region of RB1 gene. A gene of RB1 promoter region of approximately 2 kb was amplified with the pair of primers 5'-GAA GAT CTT TGA AAT TCC TCC TGC ACC A-3´ (Bgl.RbPro-S) and 5'-CCC AAG CTT AGC CAG CGA GCT GTG GAG-3´ (Hind.RbPro-AS), and

incorporated into PicaGene Basic vector 2 (manufactured by Toyo Ink Mfg. Co., Ltd.). Then, RB1 promoter which controls expression of firefly luciferase was used to prepare pGV-RbPro vector. The prepared pGV-RbPro vector was then retranscribed with pRL-SV40 encoding the sea pansy luciferase gene, as an internal control, and incorporated into K562 cell using LIPOFECTAMINE PLUS reagent (manufactured by GIBCO-BRL). Results of analysis conducted after 48 hours using a double luciferase assay system (Toyo Ink Mfg. Co., Ltd.) showed that K562 cell introduced with RB1CC1 gene exhibited strong luciferase activity compared to K562 cell incorporated with lac Z as a control, showing that introduction of the RB1CC1 gene enhanced the transcriptional activity of RB1 gene promoter (Figure 10).

(Example 10 Loss of heterozygosity at locus (D8S567) of RB1CC1 gene in primary breast cancer)

DNA samples of cancer tissue and genome DNA from same patients were amplified by PCR and the amplification products were analyzed using 8% urea-denatured polyacrylamide gel electrophoresis. Results obtained by silver staining after electrophoresis are shown in Figure 11. While two bands were observed for the genome DNA of each patient to indicate retention of heterozygosity, only one band was detected in five cases of DNA of cancer tissue, indicating loss of heterozygosity (Figure 11).

(Example 11 Analysis of mutation of RB1CC1 gene of the present invention in breast cancer)

Mutations of RB1CC1 gene were identified by analyzing the genetic sequence of cDNA samples that were amplified using ELONGASE System (manufactured by GIBCO-BRL) with the pair of primers (CC1-S2 and CC1-AS2) set forth in SEQ ID Nos: 6 and 25 that were used in Example 1, using ABI PRISM 310 genetic analyzer and the primers set forth in SEQ ID Nos: 7 to 24 in the sequence listing. As a result, 7 cases of mutation were verified among 35 cases of breast cancer, and 9 kinds of variants were verified. This result was reconfirmed using primers set forth in SEQ ID Nos: 38 to 52. Results are shown in Table 2.

Table2. Mutations of RB1CC1 gene in primary breast cancer

sample name	nucleotide mutation	location (exon)	predicted influence	genome DNA	State of *RB1CC1 gene* allele	protein	State of *RB1* LOH	protein
MMK3	c.11_4800del c.325_1585del	3-24 5-11	Y4fsX4 P109fsX122	wild type	plural heterozygous deletions	(-)	(-)	↓↓
MMK6	c.10_4798del c.1233_4533del	3-24 9-23	Y4fsX48 D411fsX431	wild type	plural heterozygous deletions	(-)	(-)	(-)
MMK1	c.957_4785del	7-24	N319fsX368	wild type	plural heterozygous deletions	(-)	(-)	↓↓
MMK15	c.1635_4719del	12-24	S545fsX557	wild type	plural heterozygous deletions	(-)	(-)	(-)
MMK31	c.212_4188del	5-24	I71fsX111	wild type	plural heterozygous deletions	(-)	(-)	(-)
MMK38	c.241_4621del	5-22	C81fsX99	wild type	plural heterozygous deletions	(-)	(-)	↓↓
MMK40	c.591_4678del	7-23	S197fsX212	wild type	plural heterozygous deletions	(-)	(-)	↓↓

(-): absent, ↓↓: significantly decreased
LOH: loss of heterozygosity

(Example 12)

Figure 12 shows results of analysis of PCR products for MMK6 in which mutation was observed in RB1CC1 gene and

MMK29 in which mutation was not observed among samples analyzed in Example 11, as well as the results of genetic sequence analysis corresponding thereto. It was found that a gene of 4.9 kb expressed in MMK29 that was without mutation, while the 4.9-kb expression was not observed in MMK6 with mutation and expression of gene fragments (1456 bp and 98 bp) was observed (Figure 12).

(Example 13 Analysis by Western blotting)

From the samples analyzed in Example 11, expression of the novel protein RB1CC1 and the RB1 protein was verified by Western blotting in 3 cancers (MMK6, MMK40, MMK38) in which mutation was observed in RB1CC1 gene and 2 cancers (MMK12, MMK29) in which mutation was not observed. After subjecting extracted protein to 5% SDS-polyacrylamide gel electrophoresis, and then transferring to PVDF membrane, reaction was conducted with the anti-human RB1CC1 antiserum (α-RB1CC-642) prepared in Example 4. The RB1 protein was reacted with RB1 monoclonal antibody (G3-245, manufactured by PharMingen Inc.). After reaction, detection was carried out using ECL reagent (manufactured by Amersham Biosciences). The results are shown in Figure 13. While novel protein RB1CC1 having a molecular weight of 180 kDa and RB1 protein of a molecular weight of 110 to 116 kDa both expressed in MMK12 and MMK29 without mutation, in contrast, expression of either protein was not observed in any of 3 cancers with

a mutation (Figure 13).

(Example 14 Immunohistological staining)

Immunohistological staining was conducted for 2 cancers (MMK3, MMK6) in which mutation in RB1CC1 gene was observed and 1 cancer (MMK 12) in which mutation was not observed among samples analyzed in Example 11. The antibody used for reaction was the same as that in Example 13, and the antibody was reacted with tissue sections prepared from paraffin blocks obtained from each of cancer samples. As shown in Figure 14, the expression levels of novel protein RB1CC1 and RB1 protein correlated, and it was verified that expression levels were clearly lower in 2 cancers (MMK3, MMK6) in which mutation in RB1CC1 gene was observed compared to the cancer (MMK 12) in which mutation was not observed (Figure 14).

(Example 15)

54 samples of primary breast cancer tissue were assayed by immunohistological staining in a similar manner to Example 14, and the RB1CC1 protein was not detected in 8 samples (corresponding to 15%). Then, RB1 protein expression was absent or significantly lowered in all of the samples.

For 46 cases expressing RB1CC1 protein, the RB1 protein was simultaneously expressed in 45 cases. When the RB1 protein expression was compared with the RB1CC1 positive

group and negative group by stain indication using immunohistological staining (indication showing as a percentage the ratio of the number of cells stained among 1000 or more cells), the RB1CC1 positive group and negative group were found to show a positive correlation with RB1CC1 expression, with 78.6 ± 13.9% and 13.6 ± 12.1%, respectively (Figure 15a). Meanwhile, when immunohistological staining for Ki-67 was conducted using mouse monoclonal antibody (NCL-Ki-67-MMI, manufactured by Novocastra Inc.), the stain indication was 20.3 ± 12.8% for the RB1CC1 positive group and 65.0 ± 12.2% for the negative group, showing a clearly inverse correlation with RB1CC1 expression (Figure 15b).

These results indicate that in cancers in which expression of RB1CC1 protein is suppressed, the cell proliferation marker Ki-67 is expressed in large amounts, and proliferation of cancer cells flourishes. It was thus found that assaying using a combination of RB1CC1 protein and Ki-67 is useful for cancer diagnosis.

By testing for the novel gene (RB1CC1 gene) of the present invention and the protein (RB1CC1) thereof, information that is useful for the diagnosis of cancer cell proliferation and cancer can be provided.

Claims

1. A protein or polypeptide which is present in nucleus of human or animal cell and which has a transcription factor function and/or a function that can induce expression of retinoblastoma gene (RB1 gene) or a gene product thereof.

2. The human protein according to claim 1, which is a polypeptide or protein selected from a group consisting of: (1) a polypeptide or protein represented by an amino acid sequence set forth in SEQ ID No: 1 in the sequence listing; (2) a polypeptide containing an amino acid sequence comprising at least five amino acids of the amino acid sequence of the polypeptide or protein; (3) a polypeptide or protein having homology of at least approximately 70% at the amino acid sequence level with the polypeptide or protein; and (4) a protein or polypeptide having a mutation or induced mutation such as a deletion, substitution or addition of one to several amino acids relative to the amino acid sequence of the polypeptide or protein according to any one of the preceding (1) to (3).

3. The animal protein according to claim 1 that is a protein derived from mouse, and which is a polypeptide or protein selected from the group consisting of: (1) a polypeptide or protein represented by an amino acid sequence set forth

in SEQ ID No: 2 in the sequence listing; (2) a polypeptide containing an amino acid sequence comprising at least five amino acids of the amino acid sequence of the polypeptide or protein; (3) a polypeptide or protein having homology of at least approximately 70% at the amino acid sequence level with the polypeptide or protein; and (4) a protein or polypeptide having a mutation or induced mutation such as a deletion, substitution or addition of one to several amino acids relative to the amino acid sequence of the polypeptide or protein according to any one of the preceding (1) to (3).

4. A nucleic acid coding for the polypeptide or protein according to any one of claims 1 to 3, or a complementary strand thereof.

5. A nucleic acid hybridizing under stringent conditions with the nucleic acid according to claim 3 or the complementary strand thereof.

6. A nucleic acid represented by a base sequence comprising at least 15 consecutive bases of the base sequence of a nucleic acid set forth in SEQ ID Nos: 3 to 4 in the sequence listing or a complementary strand thereof, wherein a polypeptide expressed by transcription of the nucleic acid is the polypeptide according to any one of claims 1 to 3.

7. A recombinant vector containing the nucleic acid according to any one of claims 4 to 6.

8. A transformant that was transformed with the recombinant vector according to claim 7.

9. A method for producing the polypeptide or protein according to any one of claims 1 to 3, comprising a step of culturing the transformant according to claim 8.

10. Nucleic acid primers set forth in SEQ ID Nos: 5 to 132 in the sequence listing, which hybridize under stringent conditions with the nucleic acid according to any one of claims 4 to 6 or the complementary strand thereof.

11. An antibody that immunologically recognizes the polypeptide or protein according to any one of claims 1 to 3.

12. A method of screening for compounds that inhibit or enhance a function that can induce transcription factor activity and/or expression of RB1 gene of the polypeptide or protein according to any of claims 1 to 3, wherein the method uses at least one member of the group consisting of the polypeptide or protein according to any one of claims

1 to 3 and the antibody according to claim 11.

13. A method of screening for compounds that interact with the nucleic acid according to claim 4 or 6 to inhibit or enhance expression of the nucleic acid, wherein the method uses at least one member of the group consisting of the nucleic acid according to any one claims 4 to 6, the vector according to claim 7, the transformant according to claim 8, and the nucleic acid primers according to claim 10.

14. A compound that was screened by the screening method according to claim 12 or 13.

15. A compound that inhibits or enhances transcription factor activity and/or a function that can induce expression of RB1 gene of the polypeptide or protein according to any of claims 1 to 3.

16. A compound that interacts with the nucleic acid according to any one of claims 4 to 6 to inhibit or enhance expression of the nucleic acid.

17. A pharmaceutical composition for use in treatment of multidrug resistance that is resistance to treatment with anticancer agents, wherein the pharmaceutical composition comprises at least one member of the group consisting of

the polypeptide or protein according to any of claims 1 to 3, the nucleic acid according to any one of claims 4 to 6, the vector according to claim 7, the transformant according to claim 8, the nucleic acid primers according to claim 10, the antibody according to claim 11, and the compound according to any one of claims 14 to 16.

18. A method of testing and diagnosing a disease related with expression or activity of the polypeptide or protein according to any of claims 1 to 3, wherein the method comprises a step of conducting analysis employing (a) a nucleic acid encoding the polypeptide or protein and/or (b) the polypeptide or protein, as a marker in a sample.

19. The method of testing and diagnosing according to claim 18, which is a method of testing cancer cells or a method for diagnosing a cancer.

20. The method according to claim 18 or 19 which detects expression, increase, decrease, lack or the like of all or a part of the polypeptide or protein according to any of claims 1 to 3, wherein the method uses the antibody according to claim 11.

21. The method according to claim 18 or 19 which detects expression, mutation, lack or insertion or the like of all

or a part of a gene encoding the polypeptide or protein according to any of claims 1 to 3 through a step of amplifying a gene encoding the polypeptide or protein according to any of claims 1 to 3 using at least one of nucleic acid primers according to claim 10.

22. The method according to any of claims 18 to 21, wherein the method combines assay of expression, increase, decrease, mutation, lack or insertion or the like of all or a part of tumor-suppressor gene retinoblastoma gene (RB1 gene) or the gene product thereof (RB1 protein).

23. The method according to any of claims 18 to 22, wherein the method combines assay of expression, increase, decrease, mutation, lack or insertion or the like of all or a part of multidrug resistance gene (MDR1 gene) or the gene product thereof (MDR1 protein: P-glycoprotein).

24. The method according to any of claims 18 to 23, wherein the method combines assay of expression, increase, or decrease or the like of all or a part of the cell proliferation marker, Ki-67 protein.

25. A method that tests drug sensitivity of a cancer cell using the method according to claim 23.

26. A kit and a reagent for assay or diagnosis, for use in the method according to any of claim 18 to 25.

ABSTRACT

To provide a novel gene and protein involved in multidrug resistance in cancer, to elucidate functions of the gene and protein, to provide methods of detecting the gene and antibody against the protein and of testing and diagnosing cancer using the gene and antibody, we found a novel protein (RB1CC1) or polypeptide and gene thereof present in nucleus of human or animal cells and having transcription factor functions and/or functions inducing expression of retinoblastoma-1 gene (RB1 gene) or the gene product. We determined the amino acid sequence and cDNA sequence, conducted gene amplification and detection with primers hybridizing with the gene, tested for expression and mutation of the gene, discovered the gene relates to cancer cell proliferation and assayed cancers, prepared antibody against the protein and detected the protein using the antibody, whereby we found a relation between the protein and cancer cell proliferation, and assayed cancers.

Drawings

Fig. 1

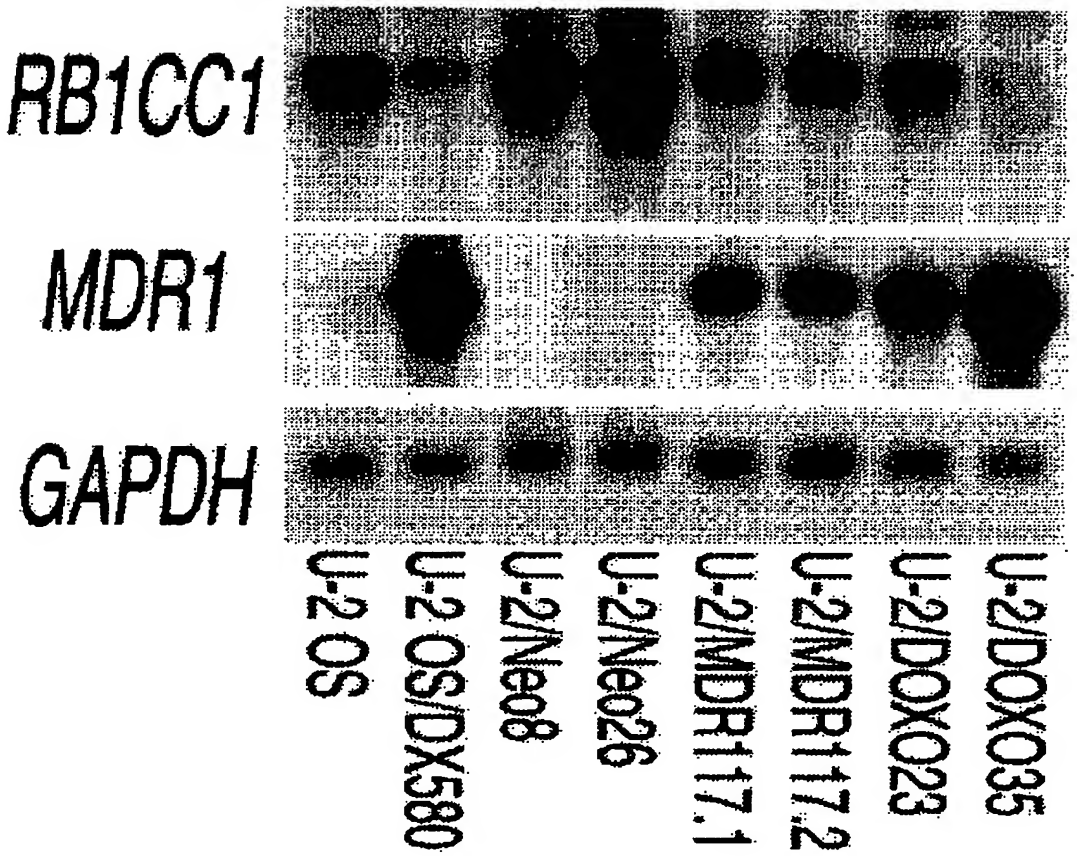


Fig. 2

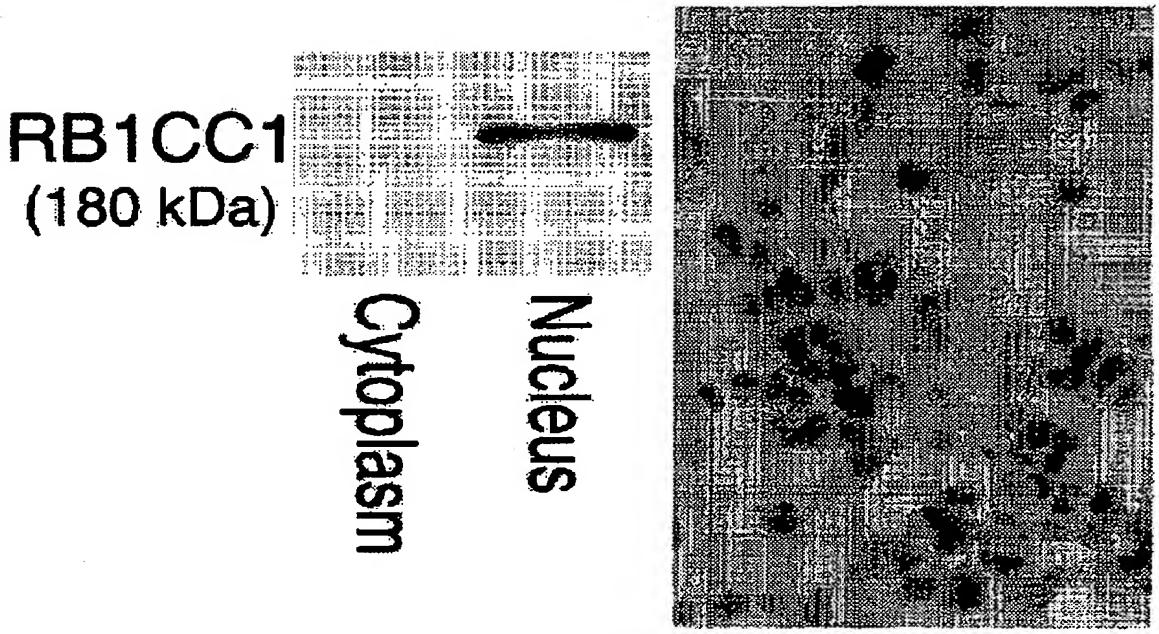
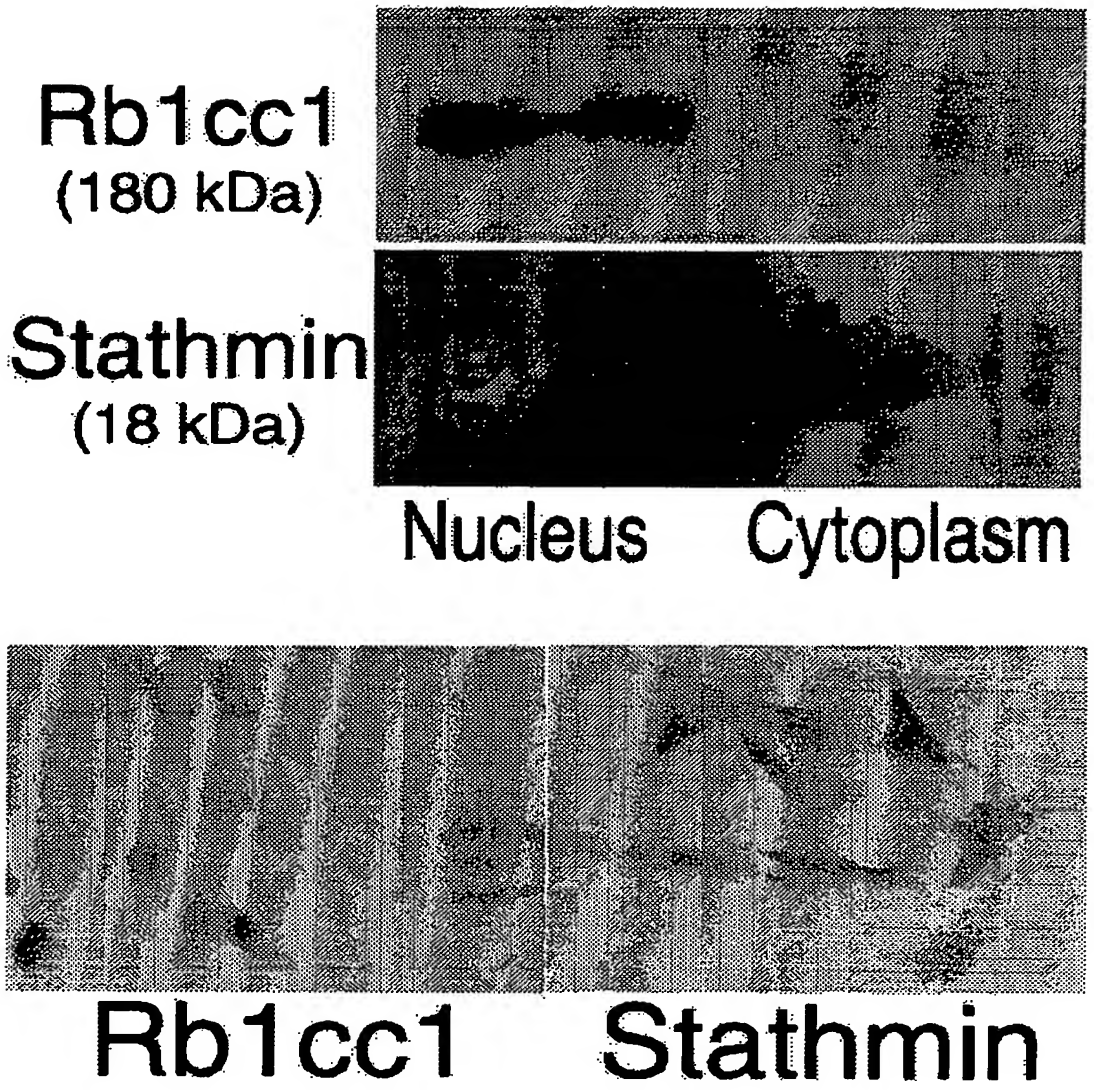


Fig. 3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(180 kDa)
Stathmin
(18 kDa)
Nucleus
Cytoplasm
Rb1cc1
Stathmin

Fig. 4

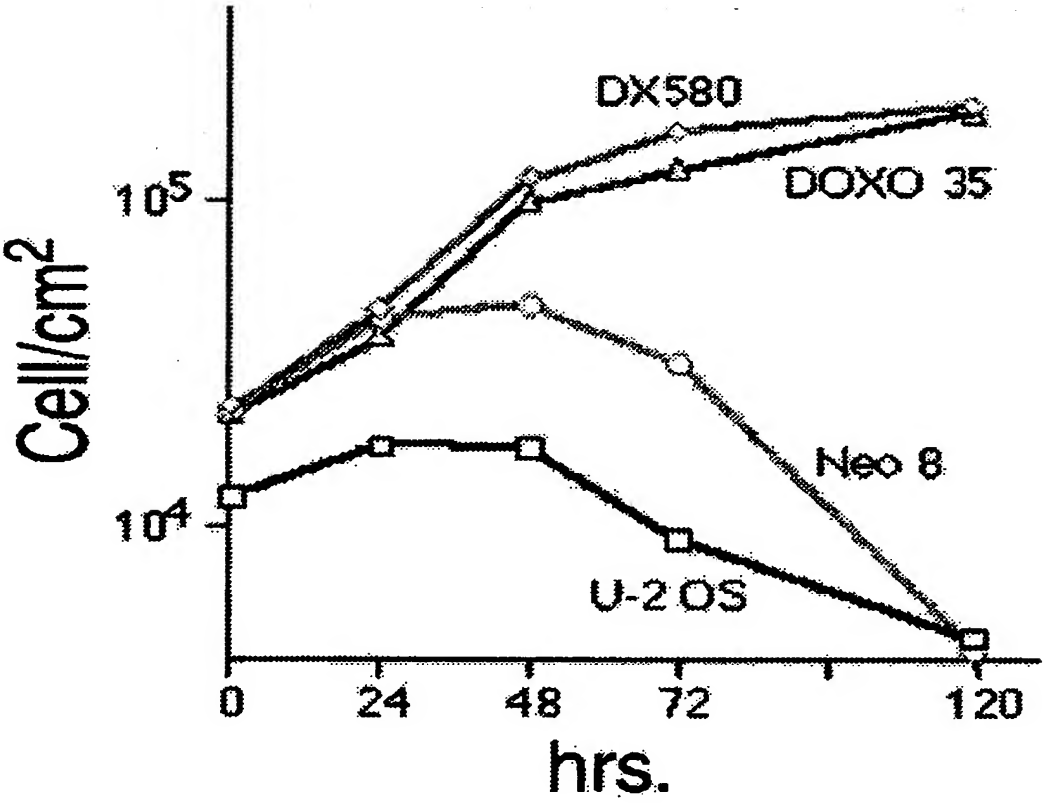
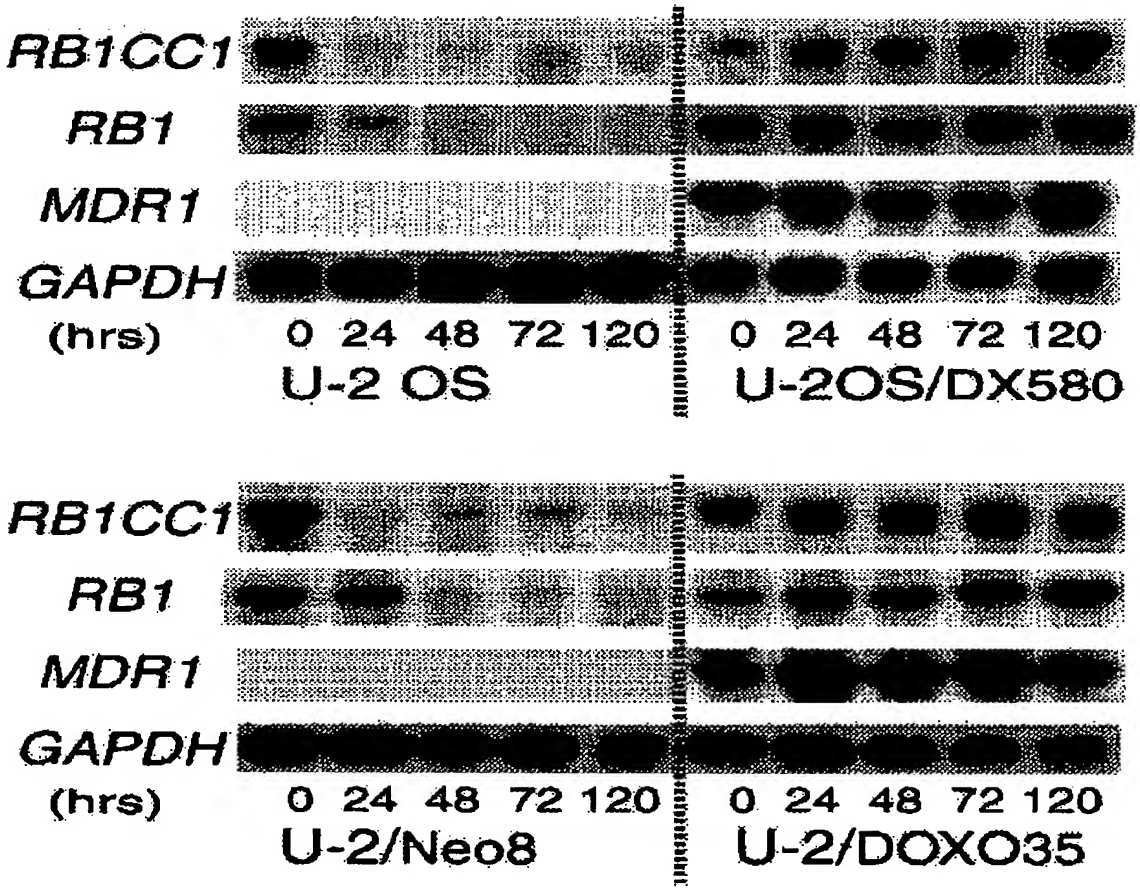


Fig. 5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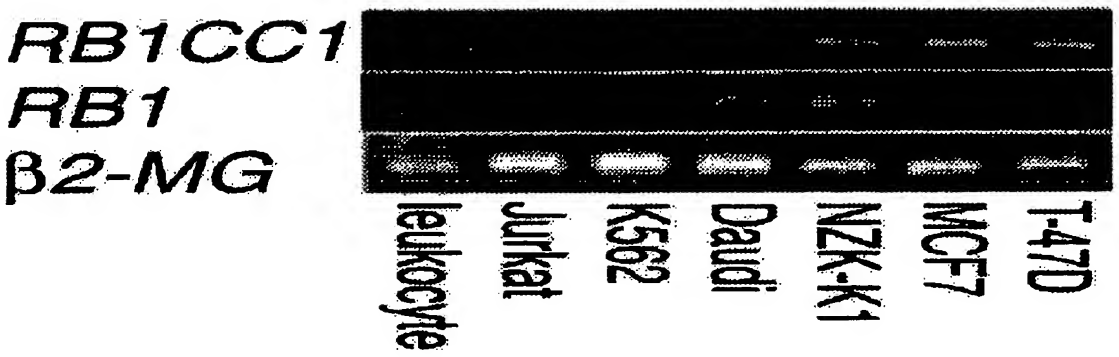
RB1
MDR1
GAPDH
(hrs)
0 24 48 72 120
0 24 48 72 120
U-2 OS
U-2OS/DX580
RB1CC1
RB1
MDR1
GAPDH
(hrs)
0 24 48 72 120
0 24 48 72 120
U-2/Neo8
U-2/DOXO35

Fig. 6


RB1CC1
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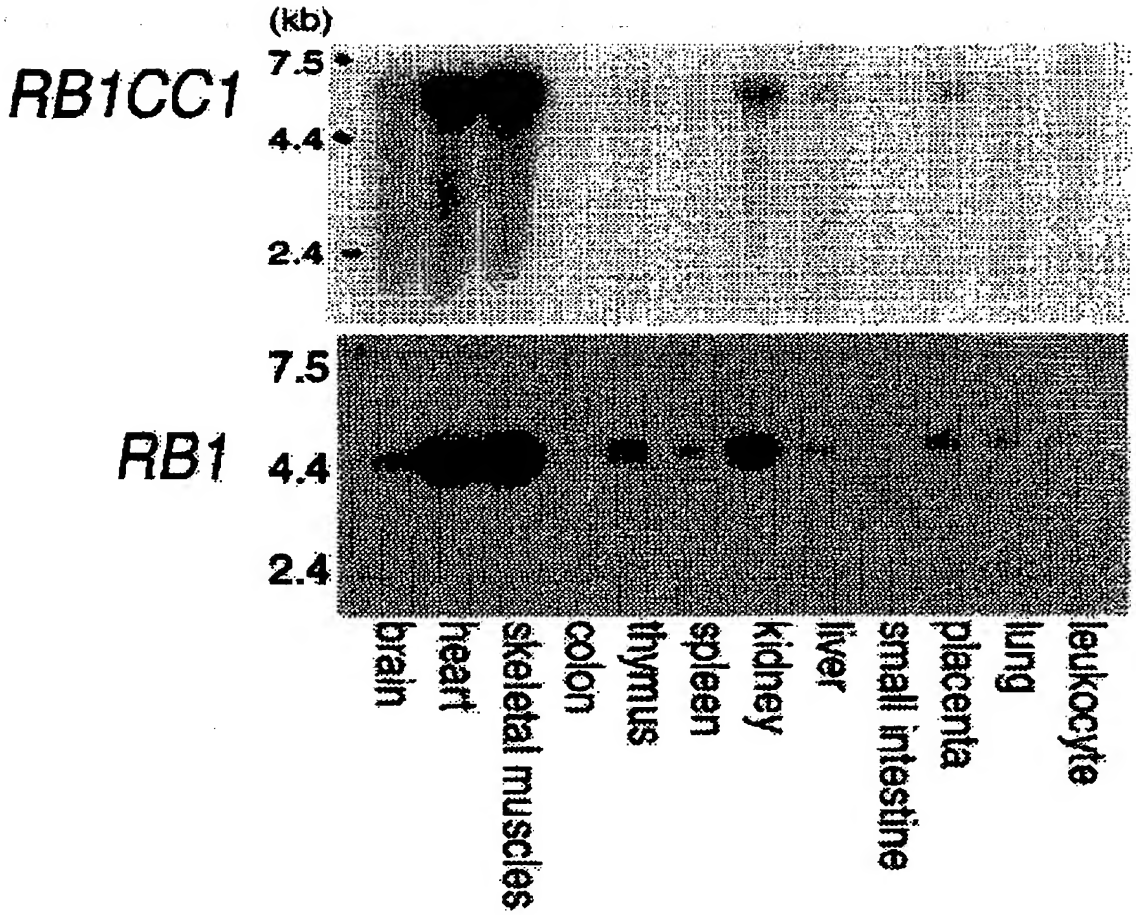
β2-MG
leukocyte
Jurkat
K562
Daudi
NZK-K1
MCF7
T-47D

Fig. 7


(kb)
RB1CC1
7.5
4.4
2.4
RB1
7.5
4.4
2.4
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heart
skeletal muscles
colon
thymus
spleen
kidney
liver
small intestine
placenta
lung
leukocyte

Fig. 8

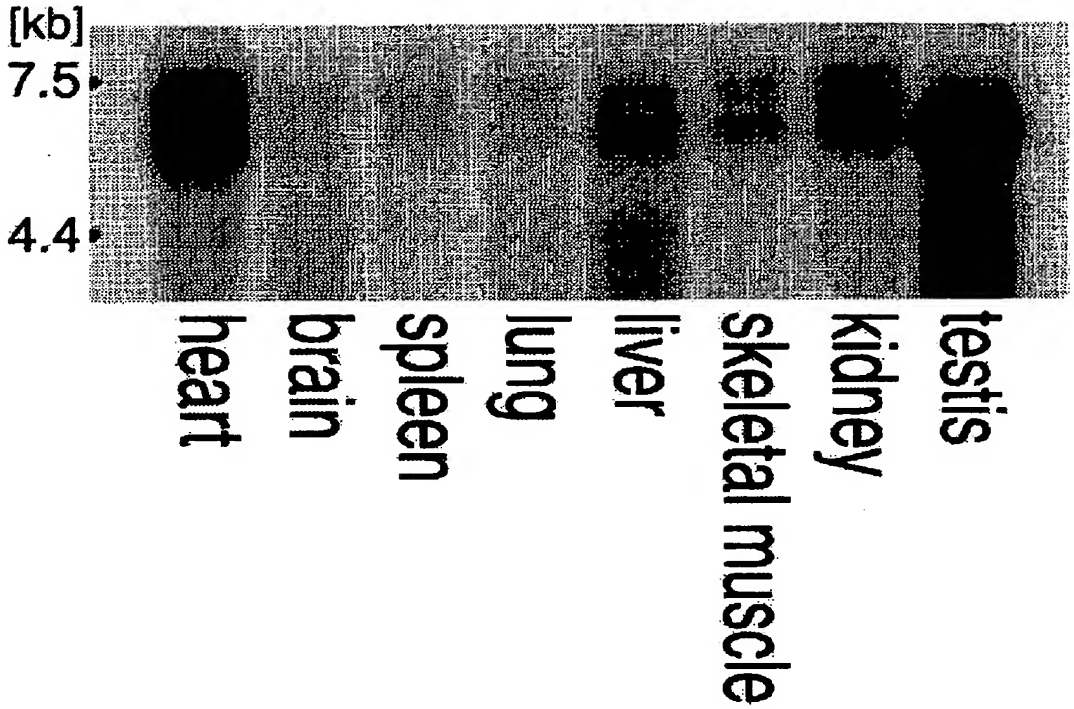
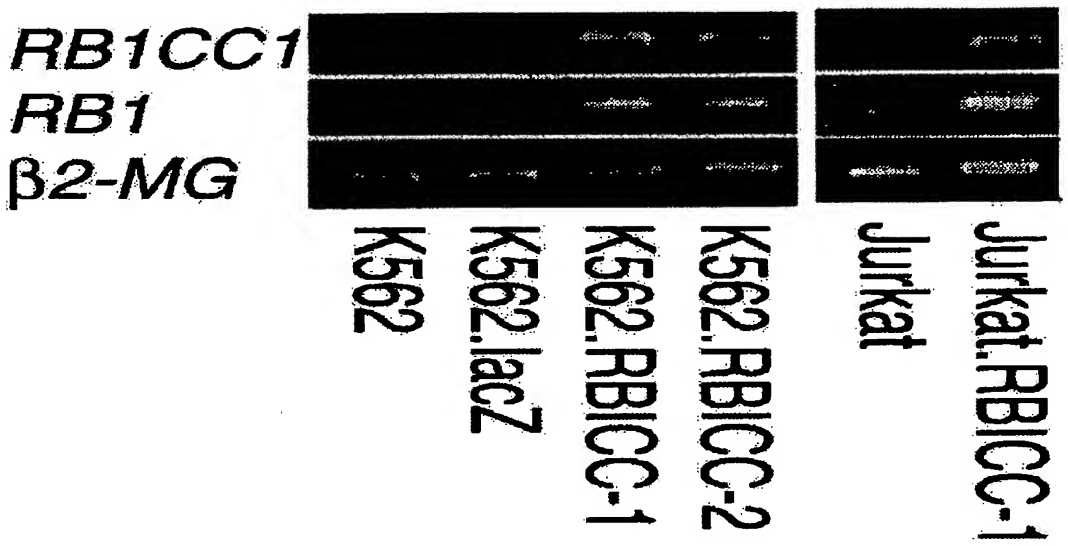

[kb]
7.5
4.4
heart
brain
spleen
lung
liver
skeletal muscle
kidney
testis

Fig. 9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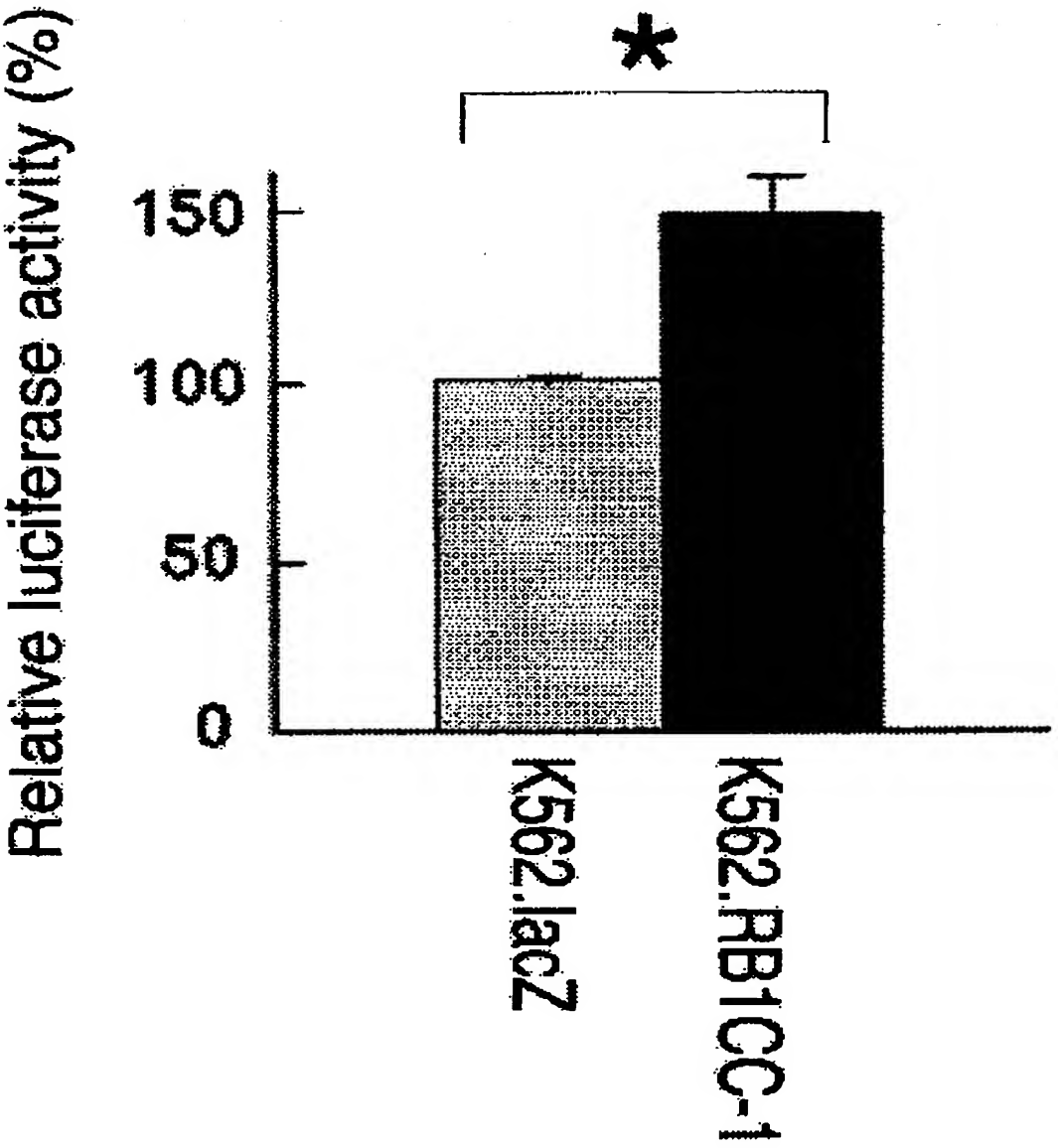
β2-MG
K562
K562.lacZ
K562.RBICC-1
K562.RBICC-2
Jurkat
Jurkat.RBICC-1

Fig. 10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150
100
50
0
*
K562.lacZ
K562.RB1CC-1

Fig. 11

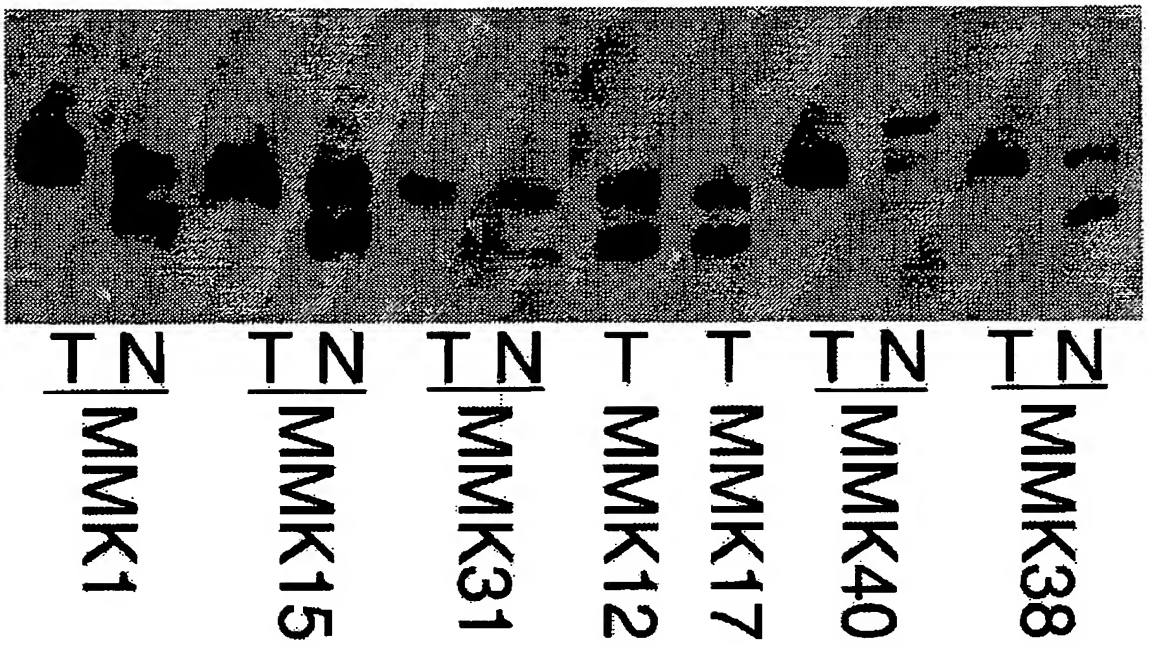
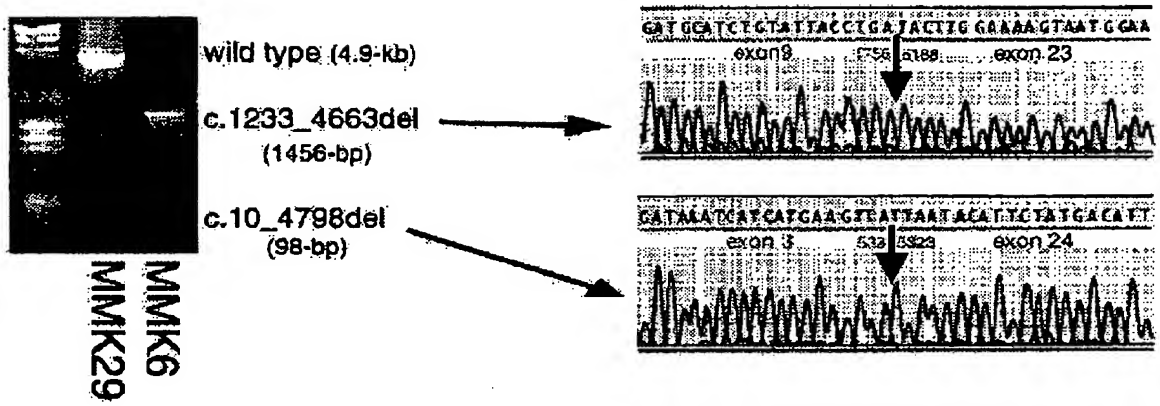


Fig. 12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(1456-bp)
c.10_4798del
(98-bp)
MMK6
MMK29
exon 9
exon 23
exon 3
exon 24

Fig. 13

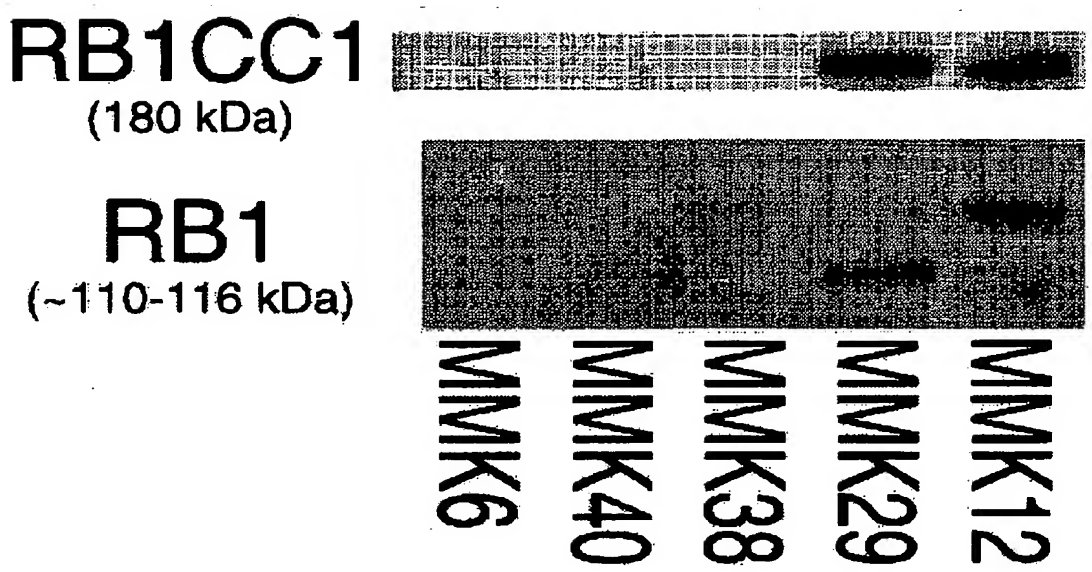


Fig. 14

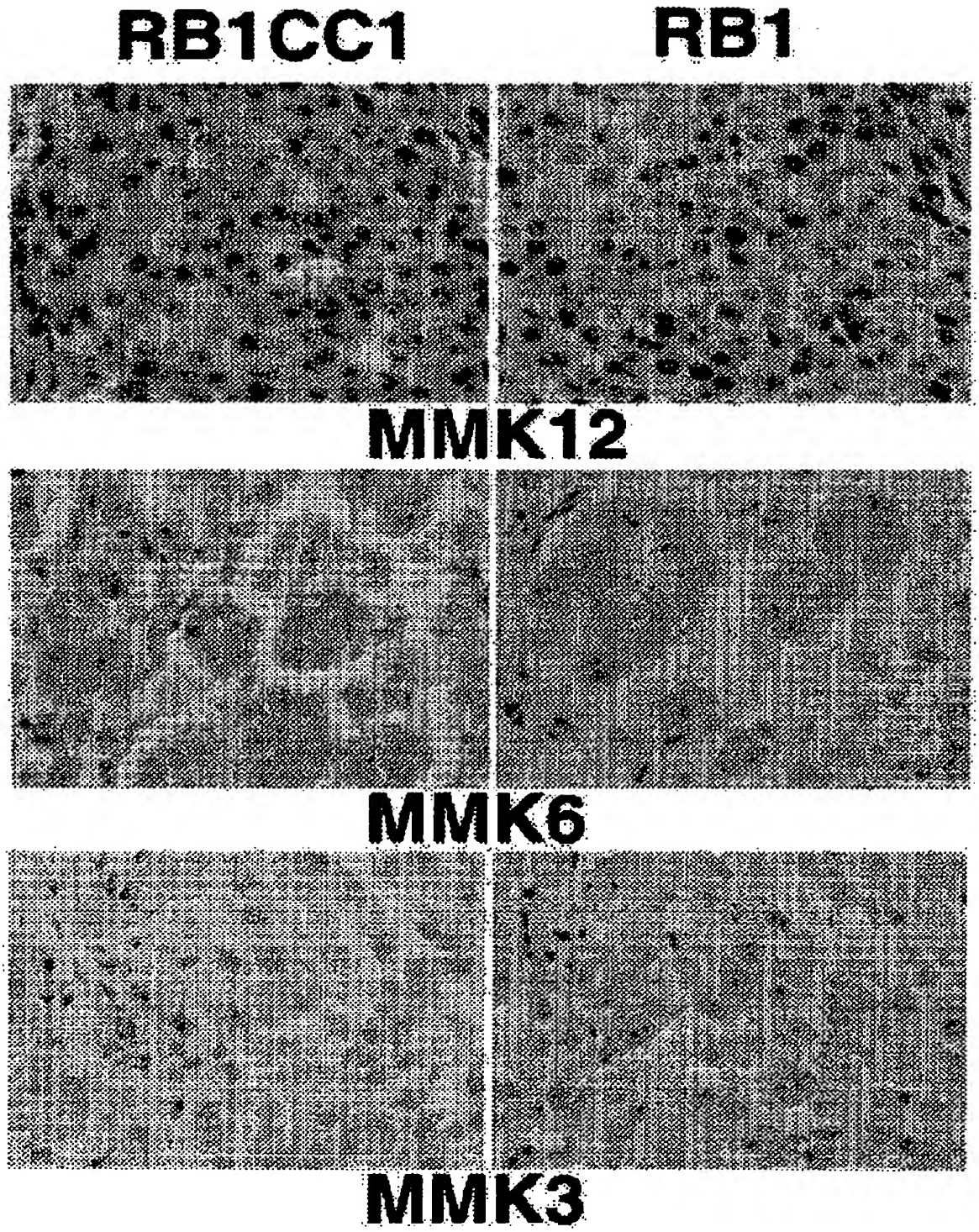
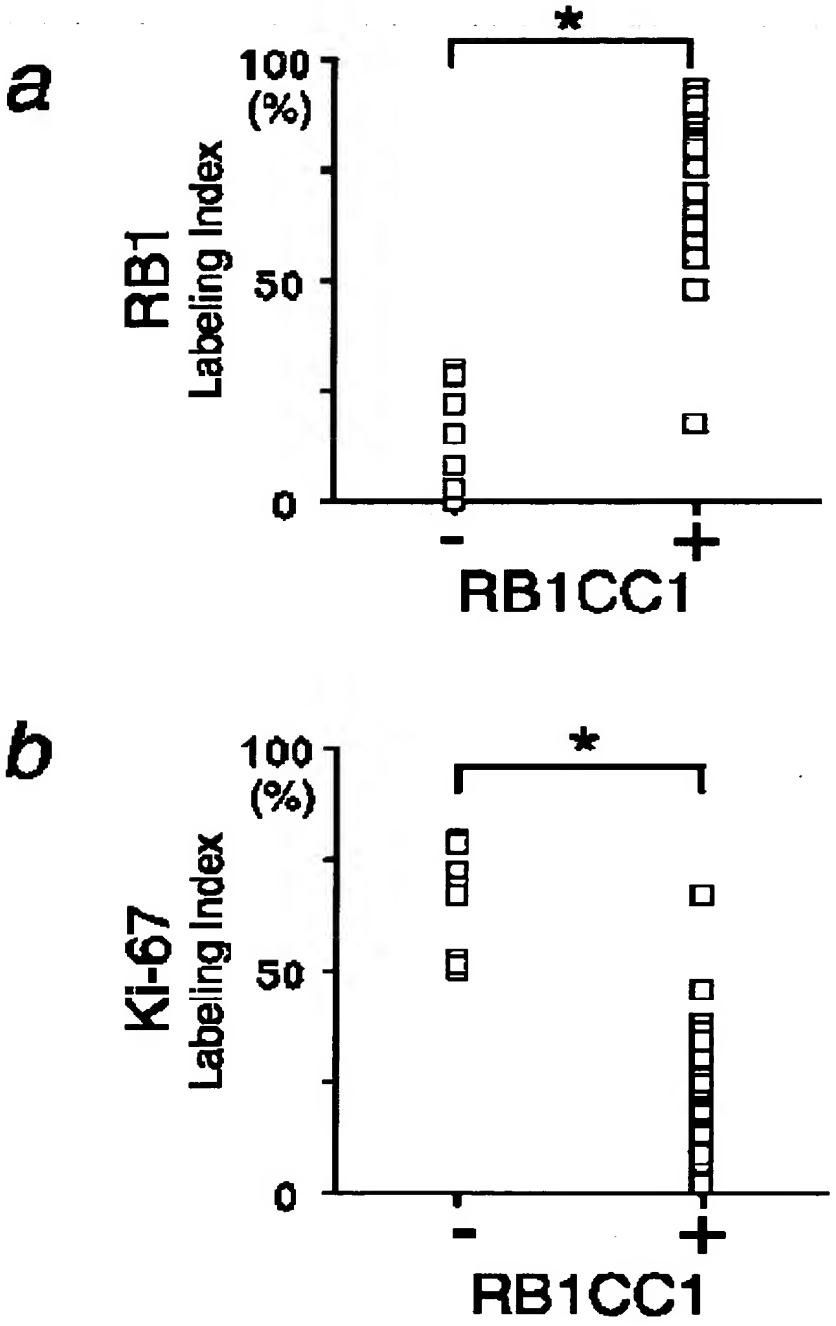


Fig. 15


a
*
100
(%)
50
0
RB1
Labeling Index
-
+
RB1CC1
b
*
100
(%)
50
0
Ki-67
Labeling Index
-
+
RB1CC1

Docket No. 3190-070

Kilyk & Bowersox, P.L.L.C.

Declaration and Power of Attorney for Patent Application
特許出願宣言書及び委任状
Japanese Language Declaration
日本語宣言書

私は、以下に記名された発明者として、ここに下記の通り宣言する：

As a below named inventor, I hereby declare that:

私の住所、郵便の宛先そして国籍は、私の氏名の後に記載された通りである。

My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明について、特許請求範囲に記載され、且つ特許が求められている発明主題に関して、私は、最初で、最先且つ唯一の発明者である（唯一の氏名が記載されている場合）か、或いは最初、最先且つ共同発明者である（複数の氏名が記載されている場合）と信じている。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

RB1 GENE INDUCED PROTEIN (RB1CC1) AND GENE

上記発明の明細書はここに添付されているが、下記の欄がチェックされている場合は、この限りでない：

the specification of which is attached hereto unless the following box is checked:

☐ _____の日に出願され、
この出願の米国出願番号またはPCT国際出願番号は、
_____であり、且つ
_____の日に補正された出願（該当する場合）

☒ was filed on January 30, 2003
as United States Application Number or
PCT International Application Number
PCT/JP03/00882 and was amended on
_____ (if applicable).
(now assigned U.S. Patent Application No. 10/516,558)

私は、上記の補正書によって補正された、特許請求範囲を含む上記明細書を検討し、且つ内容を理解していることをここに表明する。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第３７編規則１．５６に定義されている、特許性について重要な情報を開示する義務があることを承認する。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

COPY

Docket No. 3190-070

Kilyk & Bowersox, P.L.L.C.

Japanese Language Declaration
日本語宣言書

私は、ここに、以下に記載した外国での特許出願または発明者証出願、或いは米国以外の少なくとも一国を指定している米国法典第３５編第３６５条(a)によるＰＣＴ国際出願について、同第１１９条(a)(b)項又は第３６５条(b)項に基づいて優先権の利益を主張するとともに、優先権を主張する本出願の出願日よりも前の出願日を有する外国で特許出願または発明者証出願、或いはＰＣＴ国際出願については、いかなる出願も、下記の枠内をチェックすることにより示した。

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application for which priority is claimed.

Prior Foreign Application(s) 外国での先行出願		Priority Claimed 優先権主張	YES あり	NO なし
JP2002-161400 (Number) (番号)	Japan (Country) (国名)	03/06/2002 (Day/Month/YearFiled) (出願日／月／年)	☒	☐
JP2002-214978 (Number) (番号)	Japan (Country) (国名)	24/07/2002 (Day/Month/Year Filed) (出願日／月／年)	☒	☐

__他の優先権出願については添付のリスト参照

___ See attached list for additional prior foreign applications.

私は、ここに、下記のいかなる米国仮特許出願についても、その米国法典第３５編１１９条(e)項の利益を主張する。

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Application No.) (出願番号)	(Filing Date) (出願日)

私は、ここに、下記のいかなる米国出願についても、その米国法典第３５編第１２０条に基づく利益を主張し、又米国を指定するいかなるＰＣＴ国際出願についても、その同第３６５条(c)に基づく利益を主張する。また、本出願の各特許請求の範囲の主題が、米国法典第３５編第１１２条第１段に規定された態様で、先行する米国出願又はＰＣＴ国際出願に開示されていない場合においては、その先行出願の出願日と本国内出願日またはＰＣＴ国際出願日との間の期間中に入手された情報で、連邦規則法典第３７編規則１．５６に定義された特許性に関る重要な情報について開示義務があることを承認する。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況 ： 特許許可、係属中、放棄)
(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況 ： 特許許可、係属中、放棄)

私は、ここに表明された私自身の知識に係わる陳述が真実であり、且つ情報と信ずることに基づく陳述が、真実であると信じられることを宣言し、さらに、故意に虚偽の陳述などを行った場合は、米国法典第１８編第１００１条に基づき、罰金または拘禁、若しくはその両方により処罰され、またそのような故意による虚偽の陳述は、本出願またはそれに対して発行されるいかなる特許も、その有効性に問題が生ずることを理解した上で陳述が行われたことを、ここに宣言する。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

COPY

Docket No. 3190-070

Kilyk & Bowersox, P.L.L.C.

Declaration and Power of Attorney for Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書

委任状： 私は本出願を審査する手続を行い、且つ米国特許商標庁との全ての業務を遂行するために、記名された発明者として、下記の弁護士及び／または弁理士を任命する。

33432

米国特許商標庁

全ての通信は下記の住所へ送付されたい。

Kilyk & Bowersox P.L.L.C.
53 リーの東の通り
Warrenton の VA 20186

POWER OF ATTORNEY; As a named inventor, I hereby appoint The following attorney(s) and/or agent(s) to prosecute this Application and transact all business in the Patent and Trademark Office connected therewith.

33432

PATENT TRADEMARK OFFICE

Please direct all communications to the following address:

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Signature　Date 04 Dec 13

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発明者の署名　日付

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Citizenship
Japanese

郵便の宛先

Post Office Address
Same as above

Date: January 25, 2005 Label No. EV567259572US I hereby certify that, on the date indicated above, I deposited this paper with identified attachments and/or fee with the U.S. Postal Service and that it was addressed for delivery to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 by "Express Mail Post Office to Addressee" service.

Donald S. Prater /Kim Blum/ Donald S. Prater Kim Blum
Name (Print) Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Chano et al.	Examiner:	Unassigned
Application No.:	10/516,558	Group Art Unit:	Unassigned
Filed:	November 30, 2004	Confirmation No.:	Unassigned
Docket No.:	3190-070	Customer No.:	33432

For: RB1 GENE INDUCED PROTEIN (RB1CC1) AND GENE

PRELIMINARY AMENDMENT COPY

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

January 25, 2005

Sir:

Prior to examination of the above-identified application on the merits, applicants respectfully request that the application be amended as follows:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 3 of this paper.

Remarks/Arguments begin on page 9 of this paper.

COPY

Amendments to the Specification:

On page 1, after the title, please insert the following paragraph:

This application is a National Stage Application of PCT/JP03/00882, filed January 30, 2003.

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Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

1. (original) A protein or polypeptide which is present in nucleus of human or animal cell and which has a transcription factor function and/or a function that can induce expression of retinoblastoma gene (RB1 gene) or a gene product thereof.

2. (original) The human protein according to claim 1, which is a polypeptide or protein selected from a group consisting of: (1) a polypeptide or protein represented by an amino acid sequence set forth in SEQ ID No: 1 in the sequence listing; (2) a polypeptide containing an amino acid sequence comprising at least five amino acids of the amino acid sequence of the polypeptide or protein; (3) a polypeptide or protein having homology of at least approximately 70% at the amino acid sequence level with the polypeptide or protein; and (4) a protein or polypeptide having a mutation or induced mutation such as a deletion, substitution or addition of one to several amino acids relative to the amino acid sequence of the polypeptide or protein according to any one of the preceding (1) to (3).

3. (original) The animal protein according to claim 1 that is a protein derived from mouse, and which is a polypeptide or protein selected from the group consisting of: (1) a polypeptide or protein represented by an amino acid sequence set forth in SEQ ID No: 2 in the sequence listing; (2) a polypeptide containing an amino acid sequence comprising at least five amino acids of the amino acid sequence of the polypeptide or protein; (3) a polypeptide or protein having homology

of at least approximately 70% at the amino acid sequence level with the polypeptide or protein; and (4) a protein or polypeptide having a mutation or induced mutation such as a deletion, substitution or addition of one to several amino acids relative to the amino acid sequence of the polypeptide or protein according to any one of the preceding (1) to (3).

4. (currently amended) A nucleic acid coding for the polypeptide or protein according to ~~any one of claims 1 to 3~~ claim 1, or a complementary strand thereof.

5. (original) A nucleic acid hybridizing under stringent conditions with the nucleic acid according to claim 3 or the complementary strand thereof.

6. (currently amended) A nucleic acid represented by a base sequence comprising at least 15 consecutive bases of the base sequence of a nucleic acid set forth in SEQ ID Nos: 3 to 4 in the sequence listing or a complementary strand thereof, wherein a polypeptide expressed by transcription of the nucleic acid is the polypeptide according to ~~any one of claims 1 to 3~~ claim 1.

7. (currently amended) A recombinant vector containing the nucleic acid according to ~~any one of claims 4 to 6~~ claim 4.

8. (original) A transformant that was transformed with the recombinant vector according to claim 7.

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9. (currently amended) A method for producing the polypeptide or protein according to ~~any one of claims 1 to 3~~ claim 1, comprising a step of culturing the transformant with the recombinant vector containing nucleic acid coding for the polypeptide or protein ~~according to claim 8~~.

10. (currently amended) Nucleic acid primers set forth in SEQ ID Nos: 5 to 132 in the sequence listing, which hybridize under stringent conditions with the nucleic acid according to ~~any one of claims 4 to 6~~ claim 4 or the complementary strand thereof.

11. (currently amended) An antibody that immunologically recognizes the polypeptide or protein according to ~~any one of claims 1 to 3~~ claim 1.

12. (currently amended) A method of screening for compounds that inhibit or enhance a function that can induce transcription factor activity and/or expression of RB1 gene of the polypeptide or protein according to ~~any of claims 1 to 3~~ claim 1, wherein the method utilizes ~~uses at least one member of the group consisting of~~ the polypeptide, ~~or~~ the protein, or an antibody that immunologically recognizes the polypeptide or protein ~~according to any one of claims 1 to 3 and the antibody according to claim 11~~.

13. (currently amended) A method of screening for compounds that interact with the nucleic acid according to claim 4 ~~or 6~~ to inhibit or enhance expression of the nucleic acid, wherein the method utilizes ~~uses at least one member of the group consisting of~~ the nucleic acid ~~according to any one claims 4 to 6, the~~, a recombinant vector containing the nuclic acid ~~according to claim 7,~~

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~~the~~, a transformant that was transformed with the recombinant vector ~~according to claim 8, and the~~ or nucleic acid primers set forth in SEQ ID NOS: 5 to 132 in the sequence listing which hybridize under stringent conditions with the nucleic acid ~~according to claim 10~~.

14. (currently amended) A compound that was screened by the screening method according to claim 12 ~~or 13~~.

15. (currently amended) A compound that inhibits or enhances transcription factor activity and/or a function that can induce expression of RB1 gene of the polypeptide or protein according to ~~any of claims 1 to 3~~ claim 1.

16. (currently amended) A compound that interacts with the nucleic acid according to ~~any one of claims 4 to 6~~ claim 4 to inhibit or enhance expression of the nucleic acid.

17. (currently amended) A pharmaceutical composition for use in treatment of multidrug resistance that is resistance to treatment with anticancer agents, wherein the pharmaceutical composition comprises ~~at least one member of the group consisting of~~ the polypeptide or protein according to ~~any of claims 1 to 3~~ claim 1, ~~the~~, a nucleic acid coding for the polypeptide or protein or a complementary strand thereof ~~according to any one of claims 4 to 6, the~~, a recombinant vector containing the nucleic acid ~~according to claim 7, the~~, a transformant that was transformed with the recombinant vector ~~according to claim 8, the~~, nucleic acid primers set forth in SEQ ID NOS: 5 to 132 in the sequence listing which hybridize under stringent conditions with the nucleic acid ~~according to claim 10, the~~, an antibody that immunologically recognizes the polypeptide or

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protein ~~according to claim 11, and the~~ or a compound that interacts with nucleic acid to inhibit or enhance expression of the nucleic acid ~~according to any one of claims 14 to 16~~.

18. (currently amended) A method of testing and diagnosing a disease related with expression or activity of the polypeptide or protein according to ~~any of claims 1 to 3~~ claim 1, wherein the method comprises a step of conducting analysis employing (a) a nucleic acid encoding the polypeptide or protein and/or (b) the polypeptide or protein, as a marker in a sample.

19. (original) The method of testing and diagnosing according to claim 18, which is a method of testing cancer cells or a method for diagnosing a cancer.

20. (currently amended) The method according to claim 18 ~~or 19~~ which detects expression, increase, decrease, lack or the like of all or a part of the polypeptide or protein ~~according to any of claims 1 to 3~~, wherein the method utilizes ~~uses the~~ an antibody that immunologically recognizes the polypepetide ~~according to claim 11~~.

21. (currently amended) The method according to claim 18 ~~or 19~~ which detects expression, mutation, lack or insertion or the like of all or a part of a gene encoding the polypeptide or protein ~~according to any of claims 1 to 3~~ through a step of amplifying a gene encoding the polypeptide or protein ~~according to any of claims 1 to 3 using~~ utilizing at least one of nucleic acid primers set forth in SEQ ID NOS: 5 to 132 in the sequence listing, which hybridize under stringent conditions with the nucleic acid ~~according to claim 10~~.

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22. (currently amended) The method according to ~~any of claims 18 to 21~~ claim 18, wherein the method combines assay of expression, increase, decrease, mutation, lack or insertion or the like of all or a part of tumor-suppressor gene retinoblastoma gene (RB1 gene) or the gene product thereof (RB1 protein).

23. (currently amended) The method according to ~~any of claims 18 to 22~~ claim 18, wherein the method combines assay of expression, increase, decrease, mutation, lack or insertion or the like of all or a part of multidrug resistance gene (MDR1 gene) or the gene product thereof (MDR1 protein: P-glycoprotein).

24. (currently amended) The method according to ~~any of claims 18 to 23~~ claim 18, wherein the method combines assay of expression, increase, or decrease or the like of all or a part of the cell proliferation marker, Ki-67 protein.

25. (original) A method that tests drug sensitivity of a cancer cell using the method according to claim 23.

26. (currently amended) A kit and a reagent for assay or diagnosis, for use in the method according to ~~any of~~ claim 18 ~~to 25~~.

REMARKS/ARGUMENTS

Prior to payment of the filing fees, please enter the above amendment.

No questions of new matter are raised by the above amendment. Entry of the above amendment is therefore respectfully requested.

If there are any fees due in connection with the filing of this Preliminary Amendment, please charge the fees to Deposit Account No. 50-0925. If a fee is required for an extension of time under 37 C.F.R. §1.136 not accounted for above, such extension is requested and should also be charged to our Deposit Account.

Respectfully submitted,

Luke A. Kilyk
Registration No. 33,251

Attorney Docket No. 3190-071
KILYK & BOWERSOX, P.L.L.C.
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Date: January 25, 2005 Label No. EV567259572US I hereby certify that, on the date indicated above, I deposited this paper with identified attachments and/or fee with the U.S. Postal Service and that it was addressed for delivery to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 by "Express Mail Post Office to Addressee" service.

Donald S. Prater / Kim Blum Donald S. Prater / Kim Blum
Name (Print) Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Chano et al.	Examiner:	Unassigned
Application No.:	10/516,558	Group Art Unit:	Unassigned
Filed:	November 30, 2004	Confirmation No.:	Unassigned
Docket No.:	3190-070	Customer No.:	33432

For: RB1 GENE INDUCED PROTEIN (RB1CC1) AND GENE

INFORMATION DISCLOSURE STATEMENT PURSUANT TO 37 CFR 1.97(b)

COPY

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

January 25, 2005

Sir:

The attention of the Patent and Trademark Office is hereby directed to the documents listed on the attached Form PTO-1449. Pursuant to the current United States Patent and Trademark Office rules, no copies of U.S. Patents/Patent Application Publications are provided.

This Information Disclosure Statement is being submitted before expiration of the three-month period following filing of the above-captioned application.

The above information is presented so that the Patent and Trademark Office can, in the first instance, determine any materiality thereof to the claimed invention. *See* 37 CFR 1.104(a) and 1.106(b) concerning the PTO duty to consider and use any such information. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the documents cited in the attached Form PTO-1449 be made of record therein and appear

on the first page of any patent to issue therefrom.

This submission does not represent that a search has been made or that no better art exists and does not constitute an admission that each or all of the listed documents are material or constitute "prior art." If the Examiner applies any of the documents as prior art against any claim in this application and applicant determines that the cited documents do not constitute "prior art" under United States law, applicant reserves the right to present to the office the relevant facts and law regarding the appropriate status of such documents.

Applicant further reserves the right to take appropriate action to establish the patentability of the disclosed invention over the listed documents, should one or more of the documents be applied against the claims of the present application.

It is believed that no fee is required to make this a complete and timely filing. However, if it is determined that a petition or fee is required, the Commissioner is hereby authorized to charge any fee associated with this statement to our Deposit Account No. 50-0925.

Respectfully submitted,

Luke A. Kilyk
Reg. No. 33,251

Atty. Docket No.: 3190-070
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53 A East Lee Street
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Tel.: (540) 428-1701
Fax: (540) 428-1720
Enclosures: PTO-1449, w/4 Documents

FORM PTO-1449 (REV 7-80)	Atty. Docket No. 3190-070	Application No. 10/516,558
INFORMATION DISCLOSURE STATEMENT	APPLICANT: CHANO et al.	
	Filing Date: November 30, 2004	Group Art Unit: Unassigned

U.S. PATENT DOCUMENTS

EXAMINER'S INITIALS		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE, IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	TRANSLATION YES	TRANSLATION NO
		WO 00/55174	9/21/00	WIPO			X	
		WO 00/78801 A2	12/28/00	WIPO			X	

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Chano et al., "Identification of RB1CC1, a Novel Human Gene That Can Induce RB1 in Various Human Cells," ONCOGENE, Vol. 21 (2002), pp. 1295-1298.
	Chano et al., "Isolation, Characterization and Mapping of the Mouse and Human RB1CC1 Genes," GENE, Vol. 291 (2002) pp. 29-34.

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Date: January 25, 2005 Label No. EV[illegible]7259572US I hereby certify that, on the date indicated above, I deposited this paper with identified attachments and/or fee with the U.S. Postal Service and that it was addressed for delivery to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 by "Express Mail Post Office to Addressee" service.

Donald S. Prater / Kim Blum — Donald S. Prater / Kim Blum
Name (Print) Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	CHANO et al.	Examiner:	Unassigned
Application No.:	10/516,558	Group Art Unit:	Unassigned
Filed:	November 30, 2004	Confirmation No.:	Unassigned
Docket No.:	3190-070	Customer No.:	33432

For: RB1 GENE INDUCED PROTEIN (RB1CC1) AND GENE

STATEMENT UNDER 37 C.F.R. § 1.821

COPY

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

January 25, 2005

Sir:

The diskette enclosed herewith contains a computer readable form of the Sequence Listing for the above-referenced patent application. The information recorded in computer readable form on the diskette is identical to the written sequences contained in the application as filed. The computer readable form of the sequence listing contained on this diskette is understood to comply with the requirements of 37 C.F.R. § 1.821(f). Also enclosed is a computer print-out of the sequence listing.

It is believed that no fee is required to make this complete and timely filing. However, if it is determined that a petition or fee is required, the Commissioner is hereby authorized to charge any fee associated with this statement to our Deposit Account No. 50-0925 and please consider this a petition.

Respectfully submitted,

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Okabe, Hidetoshi
Ikegawa, Shiro

<120> RB1 gene induced protein (RB1CC1) and gene

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<141> 2004-11-30

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Ser Asp Thr Arg Cys Lys Leu Glu Val Glu Leu Ala Leu Lys Glu
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Val Lys His Leu Glu Asn Gln Ile Ala Lys Ser Pro Ala Ile Asp
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Ser Thr Arg Gly Asp Ser Ser Ser Leu Val Ala Glu Leu Gln Glu
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Gly Gly Glu Cys Met Ala Ala Asp Arg Arg Val Cys Thr Tyr Ser Ala
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Gly Thr Asp Thr Asn Pro Ile Phe Leu Phe Asn Lys Glu Met Ile Leu
65                  70                  75                  80

Cys Asp Arg Ala Pro Ala Ile Pro Lys Ala Thr Phe Ser Thr Glu Asn
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Asp Met Glu Ile Lys Val Glu Glu Ser Leu Met Met Pro Ala Val Phe
            100                 105                 110

His Thr Val Ala Ser Arg Thr Gln Leu Ala Val Glu Met Tyr Asp Val
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Ala Lys Lys Leu Cys Ser Phe Cys Glu Gly Leu Val His Asp Glu His
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Leu Gln His Gln Gly Trp Ala Ala Ile Met Ala Asn Leu Glu Asp Cys
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Ser Asn Ser Tyr Gln Lys Leu Leu Phe Lys Phe Glu Ser Ile Tyr Ser
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Glu Ser Leu Val Arg Lys Cys Phe Asp Ser Met Ser Arg Leu Asp Pro
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Tyr Ala Leu Asp Gln Met Ile Ala Ser Cys Ser Arg Leu Val Asn Glu
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Thr Ser Ile Lys His Glu Lys Asp Ala Ile Val Cys Val Gln Gln Glu
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Lys Asp Gln Lys Leu Leu Glu Met Glu Lys Ile Met His Thr Gln His
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Cys Glu Ile Lys Glu Leu Lys Gln Ser Arg Glu Met Ala Leu Glu Asp
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Cys Lys  Leu Glu Val Glu Leu  Ala Leu Lys Glu Ala  Glu Thr Asp
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<400> 69
acgagctcgg tcctcagaga 20

<210> 70
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tcaggtggga gatttggttc 20

<210> 71
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<400> 71
tgccgctcat ctaggatgat 20

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cagcactgga ggacaaatca 20

<210> 73
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<400> 73
agtcacaagc cacagtgcag 20

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tgctttgaat ggcatgctta 20

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cctcaccctc cagtgttgat 20

<210> 76
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cctccgcacc atcttctg 18

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cagggtcccc gtaggact 18

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tcaggtggga gatttggttc 20

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tcggaattca tggttgacct 20

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tttcccagaa atcacgcaat 20

<210> 83
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<400> 83
tctttgttca gtgccagctt t 21

<210> 84
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accaggtggg tttgtcagag 20

<210> 85
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<400> 85
cttggcgatg caaagatgta 20

<210> 86
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<212> DNA
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<220>
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<400> 86
acactggagg gtgaggtgtc 20

<210> 87
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<400> 87
gtgtcaaatg tcagcgtggt 20

<210> 88
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gacggttgtg tcggttgg 18

<210> 89
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ttggcaactg agtggcatta 20

<210> 90
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tgccactcag ttgccaagta 20

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cacagtatct ggcggtaagt ca 22

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cccagcgctg taagtacaca 20

<210> 93
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taagcatgcc attcaaagca 20

<210> 94
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caggtgcacg gtcacataag 20

<210> 95
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gcagttttcc acactgttgc 20

<210> 96
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<220>
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<400> 96
ctccagattg gccatgattg 20

<210> 97
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<400> 97
gccaatctgg aggactgttc 20

<210> 98
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<400> 98
agaatccggt cttcccaaac 20

<210> 99
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cccaatgatg tggaatctct g 21

<210> 100
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<210> 101
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<400> 101
aagatcggct gtatgcctt 19

<210> 102
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caacagtttt ctgtggtttt gc 22

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ggatgcatct gtgttacctg a 21

<210> 104
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<220>
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<400> 104
gcctgcagtt tttctccatc 20

<210> 105
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gctccgcctt gtaatagagc 20

<210> 106
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ccaaaggatt ccctttttga 20

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gggctggtgc tttagtcaaa 20

<210> 108
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<400> 108
aaaatgagga aggccaggag 20

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<400> 109
actcctggcc ttcctcattt 20

<210> 110
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tgaggaatgg ctgcacttct 20

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<210> 112
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<210> 113
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<210> 114
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<400> 114
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<400> 115
ccacgagcaa gacaaagaac 20

<210> 116
<211> 21
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<210> 117
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tgaggaaatg caaaatgtca 20

<210> 118
<211> 20
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<400> 118
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<210> 119
<211> 22
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<400> 119
aaagtacaat gcagcagcaa ga 22

<210> 120
<211> 21
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<400> 120
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<210> 121
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<400> 121
agctctatgg tgcgtgtgc 19

<210> 122
<211> 21
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<210> 123
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<210> 124
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<210> 125
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<220>
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cattgcagtt gaaagaagaa gaaa 24

<210> 126
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<220>
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<400> 126
tgccttgaag agactgagga c 21

<210> 127
<211> 22
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<400> 127
tctcttcaag gcattctgaa aa 22

<210> 128
<211> 20
<212> DNA
<213> Artificial

<220>
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<400> 128
atccagggca ggaagagact 20

<210> 129
<211> 20
<212> DNA
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agcggcacga caattatgta 20

<210> 130
<211> 22
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<400> 130
ttttccatta ctttcccaag ga 22

<210> 131
<211> 20
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<400> 131
ctgggtcctt gggaaagtaa 20

<210> 132
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<400> 132
cagcactgga ggacaaatca 20